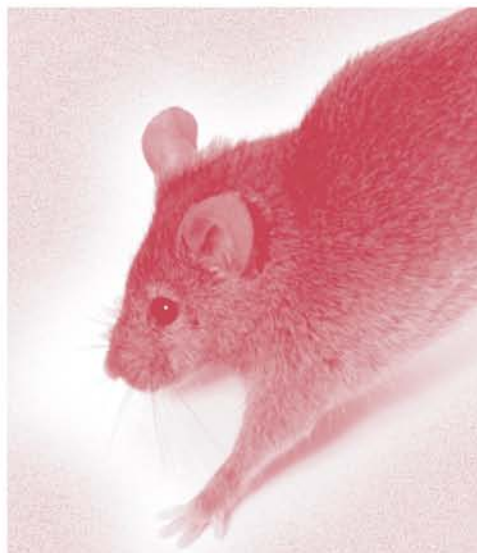


Handbook of Laboratory Animal Science

Second Edition

Volume III

Animal Models



Edited by
Jann Hau and Gerald L. Van Hoosier, Jr.

 **CRC PRESS**

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Preface

The *Handbook of Laboratory Animal Science, Second Edition: Animal Models, Volume III* is the final volume of the Handbook. Like Volume II, it is dedicated to the use of laboratory animals as models for humans in a range of biomedical research disciplines. Although Volumes II and III aim to give a comprehensive introduction to the use of animal models in biomedical research, there will be areas of research not covered by this Handbook. We welcome comments from readers and colleagues that may assist us when correcting errors and updating the text when the time comes.

We are most grateful that so many eminent scientists who are leaders in their respective fields agreed to contribute to this book with their knowledge and hands-on experience from their own research.

The individual chapters of Volume III are focused on the use of animal models in very different research areas. We hope they will be found useful for other specialists in the respective subjects as well as for postgraduate students embarking on their studies. Each chapter can be read as a stand-alone text without the necessity to consult other chapters for information. However, the entire Handbook is an integrated text with respect to giving an in-depth introduction to laboratory animal science. Volume I is focused on basic laboratory animal science — the value of animals as sensitive research tools and the importance of safeguarding the well-being of animals used in biomedical research, as well as the importance of implementing the three Rs (replacement, reduction, and refinement) in biomedical research. The chapters in Volumes II and III are all on applied laboratory animal science and comparative medicine. They do not necessarily emphasize the importance of animal welfare and implementation of the three Rs, because this principle underlying all modern use of animals in biomedical research has been thoroughly dealt with in Volume I. Biomedical postgraduate students are thus advised not just to read the most relevant chapter(s) in Volumes II and III but also to peruse Volume I.

We hope that our readers will find Volume III useful, and we wish to take this opportunity to thank the many readers of Volumes I and II for their constructive and kind comments. We also wish to thank John Sulzycki and his expert team at CRC Press for their great enthusiasm and help in the process of producing this Handbook.

Jann Hau and Gerald L. Van Hoosier, Jr.

About the Editors

Jann Hau is Professor of Comparative Medicine at the University of Uppsala in Sweden. Dr. Hau graduated in experimental biology from the University of Odense in Denmark after medical and biology studies in 1977 and specialized in laboratory animal science. Following research fellowships at the University of Odense, he obtained his doctorate (Dr. Med.) at this university. In 1983, he joined the Department of Pathology at The Royal Veterinary and Agricultural University (RVAU) in Copenhagen as associate professor and head of the Laboratory Animal Science Unit. He was later head of the Department of Pathology and dean of the Faculty of Animal Husbandry and Veterinary Science at the RVAU.

In 1991, he moved to the Royal Veterinary College (RVC) in London as professor in the London University Chair in Laboratory Animal Science and Welfare. At the RVC, he was responsible for undergraduate and postgraduate teaching in laboratory animal science and welfare, which included a specialist Master of Science course in laboratory animal science that attracted a number of postgraduate students from many parts of the world.

In 1996, Dr. Hau was appointed professor in Comparative Medicine in Uppsala. In Uppsala, he has established a number of courses for undergraduate and postgraduate students, including specialist education programs. In 2004, Dr. Hau took up a chair in Comparative Medicine and also heads the Department of Experimental Medicine at the University of Copenhagen in Denmark.

Dr. Hau has organized several international meetings and courses on laboratory animal science. He is the editor-in-chief of the *Scandinavian Journal of Laboratory Animal Science*, editor of the laboratory animals' section of the UFAW journal *Animal Welfare*, and a member of the editorial board of *In Vivo*. He is a member of a number of laboratory animal science organizations and former president of the Scandinavian Society of Laboratory Animal Science and the Federation of European Laboratory Animal Science Associations.

Dr. Hau has supervised many postgraduate Master's and Ph.D. students and published several hundred scientific papers and chapters in books. Together with Dr. P. Svendsen, he wrote the first Danish textbook on laboratory animals and animal experiments published in 1981, 1985, and 1989, and they co-edited the first edition of the *CRC Handbook of Laboratory Animal Science*, published in 1994.

Dr. Hau's current research interests include development of refined laboratory animal models for studies of biological mechanisms in reproductive biology and infections as well as development of methods to assess stress and welfare in animals, in particular rodents and nonhuman primates. His research activities also include projects focused on ways to replace, reduce, and refine the use of animals in antibody production.

Gerald L. Van Hoosier, Jr., is Emeritus Professor of Comparative Medicine in the School of Medicine at the University of Washington in Seattle, Washington. Dr. Van Hoosier graduated from the College of Veterinary Medicine at Texas A&M University at College Station, Texas, in 1957 and subsequently obtained postdoctoral training in virology and epidemiology at Berkeley, California, and in pathology at Baylor College of Medicine in Houston, Texas. From 1957 to 1962, he served as a commissioned officer in the U.S. Public Health Service assigned to the biologics program at the National Institutes of Health in Bethesda, Maryland, where he focused on the development and safety evaluation of poliomyelitis and measles vaccines. Following 5 years in the Public Health Service, Dr. Van Hoosier joined the faculty of the Division of Experimental Biology at Baylor College of Medicine in Houston, Texas, and did research on the role of viruses in the etiology of cancer. In 1969, he moved to Pullman, Washington, where he was a faculty member in the Department of Veterinary Pathology in the School of Veterinary Medicine and Director of Laboratory Animal Resources at Washington State University. He introduced a course on laboratory animals into the third year of the veterinary school curriculum, taught a graduate course on the

pathology of laboratory animals, and began the development of a series of audio tutorials in collaboration with the American College of Laboratory Animal Medicine. In 1975, Dr. Van Hoosier was invited to develop an experimental animal program at the University of Washington. He obtained a training grant for veterinarians from the National Institutes of Health and established the Department of Comparative Medicine, which offers a Master's degree. He served as the department chairman and attending veterinarian until 1995.

After becoming a Diplomate of the American College of Laboratory Animal Medicine in 1968, he served as President in 1977–1978. Other professional activities have included serving as Chairman of the Board of Trustees of the American Association for Accreditation of Laboratory Animal Care in 1981–1982, President of the American Association of Laboratory Animal Science in 1992, and a member of the Governing Board of the International Council for Laboratory Animal Science from 1995 to 1999. In addition to approximately 100 scientific papers, Dr. Van Hoosier was a coeditor of *Laboratory Hamsters*, one of a series of texts by the American College of Laboratory Animal Medicine, and served as editor of *Laboratory Animal Science* from 1995 to 1999. He is currently a member of the Editorial Council of the *Baltic Journal of Laboratory Animal Science* and *Animales de Experimentacion*.

He is the recipient of the Griffin Award from the American Association of Laboratory Animal Science and a Distinguished Alumni Award from the College of Veterinary Medicine at Texas A&M University.

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CHAPTER 1

Animal Models in Vaccinology

Coenraad F.M. Hendriksen

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INTRODUCTION

Vaccines belong to the category of immunobiologicals — products that are produced by or derived from a living organism. Immunobiologicals include a variety of products, such as vaccines, immunoglobulins, monoclonal antibodies, and antisera. The characteristic feature of vaccines is that these preparations are capable of inducing a specific and active immunity against an infecting agent or its toxin.¹ Vaccination is one of the most powerful and cost-effective tools in modern medicine. The worldwide immunization campaigns against a number of infectious diseases (e.g., diphtheria, tetanus, and measles) have led to substantial decreases in morbidity and mortality rates and, in the cases of smallpox and poliomyelitis, to complete and almost complete eradication, respectively. In the coming years, the importance of vaccines will continue to increase because of the emergence of antibiotic-resistant strains of bacteria such as *Mycobacterium tuberculosis*, the impact of new viral infections such as human immunodeficiency virus (HIV) and severe acute respiratory syndrome (SARS), increased biological warfare threats (e.g., smallpox and anthrax),

global travel and tourism, the high incidence and economic effects of infectious diseases in large livestock industries, and various other factors.²

Vaccination is not without controversy. Some groups oppose vaccination programs for religious reasons. Others adhere to the speculative concept that infant vaccination may stimulate allergic sensitization. It has been hypothesized that due to today's comprehensive immunization program, children would be more prone to develop allergic diseases due to a shift of the Th1/Th2 balance in the immune system.^{3,4} Although studies have been initiated to scientifically underpin this theory, no proof is yet available. The main reason that parents withhold immunizations from their children, however, is fear of vaccine-associated adverse effects. No vaccine is totally safe and totally effective, and adverse reactions have been reported with all vaccines, although products differ in the extent of their effects.⁵ Because public acceptability of immunizing children is inversely related to the extent of adverse reactions, much attention has been given to improving existing vaccines. One of the best examples is the whooping cough (pertussis) whole cell vaccine. Due to campaigns in the U.K. tabloid press in the mid-1970s, which magnified the adverse reactions of whole cell pertussis immunization out of proportion, vaccine uptake dropped sharply from 80 to 30% in the U.K. and also decreased in other countries. Later studies showed that the claimed adverse reactions were highly exaggerated.⁶ Table 1.1 summarizes the frequency of adverse reactions from postimmunization surveillance data as well as morbidity data from natural whooping cough infections. It can be seen from this table that whooping cough vaccination is highly cost effective. Nevertheless, public concern about adverse effects stimulated a renewed interest in basic research toward a safer product,⁷ ultimately resulting in the first acellular pertussis (acP) vaccine,⁸ which only includes the protective epitopes of the pertussis microorganism. The pertussis case is a good example of how public concern can affect the use of laboratory animals. Animal models played a crucial role in the development and screening of new pertussis vaccine candidates, and literally hundreds of thousands of animals were used in the development of the new product.

A traditional link exists between laboratory animals and vaccines. As far back as the end of the 19th century, vaccine research provided a major impetus for the development of animal models. Some of the animal models currently used in routine vaccine quality control are in fact slight modifications of the tests developed by Emile von Behring or Paul Ehrlich in the 1890s. A close association between laboratory animals and vaccines still exists. Animals are particularly required for vaccine development and for quality control. Few animals are currently needed for vaccine production.

This chapter focuses on the animal models used in vaccine research and testing. It provides information on technologies in vaccine development, on the historical context of animal models in vaccine research, and on characteristics of animal use in current vaccine development, production, and quality control.

Table 1.1 Benefits of Vaccination against Natural Whooping Cough

Adverse Reactions	Incidence (No. per 100,000 Cases) Following		Ratio of Infection/Vaccination
	Infection	Vaccination	
Shock	—	15	—
Convulsions	4000	45	89
Encephalitis	2000	1.5	1300
Permanent brain damage	1300	0.3	4300
Death	2000	0.2	10,000

Source: Galazka, A.M., Lauer, B.A., Henderson, R.H., and Keja, J., *Bull. WHO*, 62, 357, 1984.

VACCINE DEVELOPMENT TECHNOLOGIES

The work of Edward Jenner on smallpox is generally considered to be the first scientific approach to vaccine development. After 25 years of study, Jenner published in 1798 the results of his successful experiment in which an 8-year-old boy, James Phipps, was inoculated with cowpox material and subsequently challenged with smallpox virus. Jenner's study was entirely based on epidemiology and observation. Animal experiments did not contribute to it in any way.¹⁰ It took almost 100 years before Louis Pasteur discovered several new vaccines, against fowl cholera (1880), anthrax (1881), and rabies (1885). All these vaccines were partly developed by trial and error without full understanding of the pathogenesis of the diseases. Nevertheless, Pasteur was the first to approach vaccine development in a systematic and coherent way. The virulent rabies virus, obtained from the saliva of infected dogs and humans, was attenuated in rabbits by multiple passages of the virus in cerebral and spinal cord tissue and finally by exposure to atmospheric oxygen. After Pasteur's pioneering work, vaccination as a means of combating infectious diseases was taken up and extended by many researchers. In fact it was Pasteur who came up with the word "vaccination" as a tribute to Edward Jenner (the Latin word *vacca* means "cow"). The term "vaccinology," introduced by Jonas Salk in 1977, can be defined as "the study and the application of the requirements for effective immunization," meaning nothing other than the science of vaccines from A to Z,¹¹ thus including development, production, quality control, and research on vaccine-related issues such as adjuvants.

Figure 1.1 shows the major vaccines for human use that have been developed since Jenner's smallpox vaccine. They amount to a total of 21 products. Although the number is small, these products all have had tremendous impact on human health care. Several diseases with high morbidity and mortality rates in the 19th century, such as diphtheria and measles, are now almost unknown,

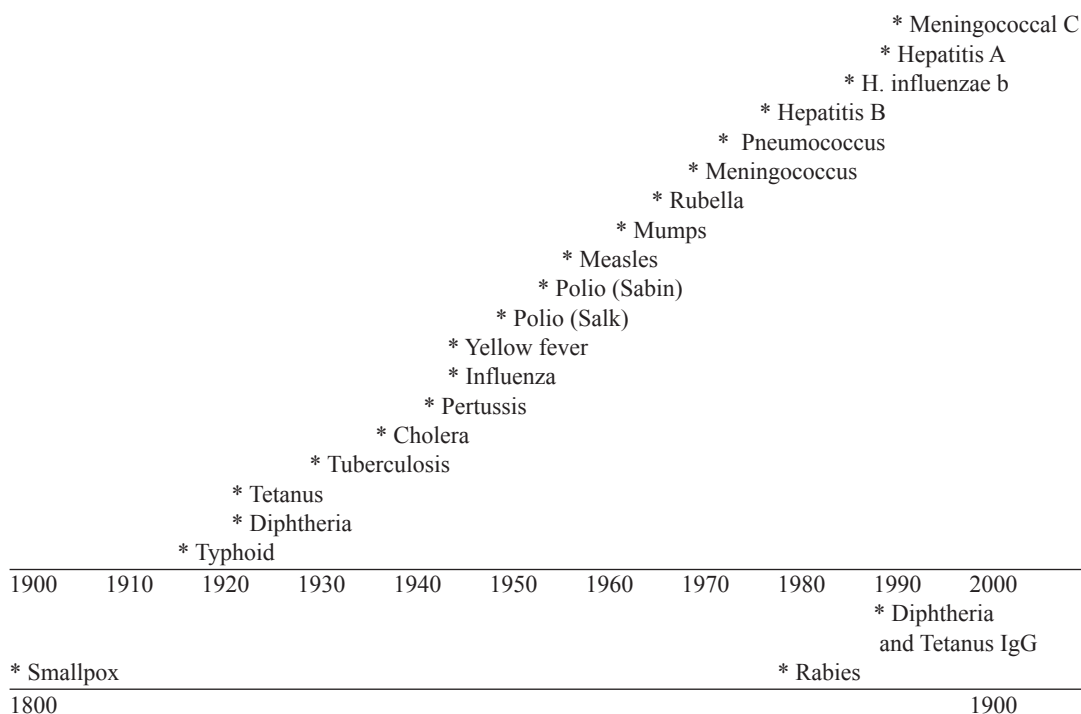


Figure 1.1 History of vaccine development. (Only vaccines for human use are shown.) (Adapted from van der Zeist, B.A.M. and Lenstra, J.A., *LAB/ACBC*, 9, 18, 1988.)

at least in Western countries, while smallpox has been eradicated and poliomyelitis is almost eradicated.¹²

Roughly, vaccine development from the early days until now can be divided in three time periods, with each era characterized by specific production technologies.

First-Generation Vaccines

These conventional products consist of attenuated or inactivated (whole) microorganisms. Pasteur's technique of attenuation was subsequently used for the production of several other vaccines, such as the oral poliomyelitis vaccine developed by Sabin in 1954 and the measles vaccine in 1958. Inactivated vaccines are produced by inactivating the whole microorganism or bacterial products (toxins) using heat or chemicals such as formaldehyde. Examples of inactivated vaccines are the whole cell pertussis vaccine, tetanus toxoid, and the inactivated poliomyelitis vaccine developed by Salk in 1953. Bacterial microorganisms have been grown on culture media, and viruses have been cultured in laboratory animals (e.g., rabies virus in rabbit or suckling mouse brains), in embryonated eggs, and after the 1950s also in cell cultures. In addition, immune response to vaccine antigens is enhanced by addition of an adjuvant product, generally aluminum salts, AlPO_4 and $\text{Al}(\text{OH})_3$. Conventional vaccines are characterized by high complexity in composition and structure. As a consequence, emphasis has to be given to extensive batch-related quality control. Vaccine batches are produced in volumes of up to 1000 L.

Second-Generation Vaccines

A rational approach to vaccine development started in the late 1950s. The approach was based on the subunit principle — the isolation and purification of only those antigenic components (antigenic epitopes) of the microorganism that underlie the protective immune response. Examples of subunit viral vaccines are the influenza vaccines based on surface protein and a subviral hepatitis B vaccine. A breakthrough in subunit vaccine development only occurred after the introduction of hybridoma technology to produce monoclonal antibodies,¹⁴ as these antibodies allowed selection and identification of protective epitopes. The acellular pertussis (acP) vaccine, including one or multiple antigenic components of the pertussis microorganism,⁸ is an example of a bacterial subunit vaccine, now used in most Western countries. A group of products of particular interest in this context is the bacterial polysaccharide vaccines, derived from capsular polysaccharides of Gram-positive or Gram-negative bacteria. Polysaccharides are poorly immunogenic — a problem that can only be overcome by chemical linkage to proteins. Examples of polysaccharide vaccines available are those against *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae*.

Third-Generation Vaccines

Advances in biotechnology and concomitant developments in the fields of molecular immunology and protein chemistry opened the door to new vaccine production strategies in the 1980s. Recombinant DNA (rDNA) technology allowed for the insertion of one or more genes from microorganisms encoding immunologically relevant proteins into host systems such as viruses (e.g., vaccinia and adenovirus), yeast, or animal cells. Thus, expression of these genes by the vectors induces immunity after administration. An example of a licensed rDNA product is the yeast- and Chinese hamster ovary (CHO)-derived recombinant hepatitis B (rHBsAg) vaccine. However, most experimentally produced rDNA (carrier) vaccines still have not passed official registration. Another approach to rDNA vaccines involves deleting those genes that encode for pathogenicity. One such product has been produced for Aujeszky disease.¹⁵ Another example is the double mutant acP vaccine.¹⁶

Advances in biochemistry have increased interest in polypeptide vaccines produced by chemical synthesis. The appeal of this approach is that it permits the manufacture of chemically well-defined products on an industrial scale. However, it has meanwhile become clear that, though the required antigenic peptides can be synthesized, it is extremely difficult to obtain the spatial configuration (glycosylation) of the peptide chains necessary for immunogenicity. To date, not one peptide vaccine has reached the stage of clinical trials.

Finally, the use of plasmid expressing recombinant genes of pathogens encoding for immune responses has become the basis of the DNA vaccine strategy. Intramuscular injection of the plasmid results in uptake by muscle cells, expression of the recombinant gene, and the elicitation of immunity against the protein product of the pathogen.¹⁷ DNA vaccination has been studied for a long list of pathogens such as influenza, rabies, and tuberculosis, but few products have entered clinical trials yet.¹⁸

Adjuvants

An integral part of vaccinology is research on adjuvant products. Adjuvants are chemicals or biomolecules which, when given in conjunction with a vaccine, enhance and/or modify immune responses. Currently, no adjuvant products exist that are absolutely safe. Consequently, safety and adjuvanticity must be balanced between obtaining maximum immune stimulation and minimum adverse effects.⁵ Since early vaccine development, research has been performed to develop effective formulations. However, the only adjuvants approved by regulatory authorities for use in humans include aluminium phosphate and aluminum hydroxide. As side effects are somewhat less a problem in the livestock industry, the range of products allowed for use in the veterinary clinic is much larger.

New vaccine production strategies have resulted in more defined but smaller antigenic structures such as subunits and peptides. These products are less immunogenic compared to whole cell vaccines. In addition, some new vaccines require modulation of immune response toward cell-mediated immunity (CMI). Unfortunately, aluminum “alum” is a weak adjuvant and stimulates mostly humoral immunity. Therefore, significant efforts are now underway to develop new generations of adjuvants. Promising new products, such as muramyl-dipeptide (MDP) or immunostimulatory complexes (ISCOM), not only induce strong antibody responses but also induce more balanced Th1/Th2 responses; they therefore might be useful for vaccines that require responses of the Th1 (CMI) type.¹⁹ Furthermore, an urgent need now exists for adjuvants that support new vaccine administration strategies, such as oral vaccines.

THE HISTORICAL ROLE OF ANIMAL MODELS IN VACCINOLOGY

In 1884, the German microbiologist Robert Koch published his book *Die Aethologie und die Bekämpfung der Tuberkulose*, which elaborated on the criteria that should be met in relating a microorganism to a given infection. These criteria, also known as Koch’s postulates, have been of historical importance regarding the role of animal models in the study of infectious diseases. Apart from the isolation of the microorganism in pure culture, the postulates stated that these pure cultures, following the introduction into a suitable animal model, should result in the typical clinical signs of the disease in the laboratory animal. Koch’s postulates gained general acceptance in microbiology and thus in vaccine development. When it was difficult to find a suitable animal model, as was the case for poliomyelitis, progress in vaccine development stagnated. By contrast, rapid results were obtained in research on diphtheria and tetanus when it was found that animal models were easily available.²⁰ As an example, a historical overview is given of the use of animal models in the production and quality control of diphtheria therapy and prophylaxis. In the 1800s, diphtheria was a highly contagious disease in young children with a mortality rate of up to around 40%. The

disease was also known as the “strangling angel of children”; infected children suffocated due to a pseudomembranous inflammation of the pharynx.

For a number of reasons diphtheria makes an interesting case. It nicely shows the crucial contribution animal models made to a major breakthrough in medical history (Table 1.2). Further, it also played a crucial role in the heated discussions about the moral status of animal experimentation that took place at the end of the 19th century. Diphtheria antitoxin and vaccine completely changed the previously hopeless treatment of a child with diphtheria. Shortly after the introduction of diphtheria antiserum, morbidity and mortality rates declined significantly. The fact that the use of animal models had made the treatment of this feared disease in young children possible convinced many of the value of this type of experimentation and undermined the criticism leveled at it. As a result, the animal welfare movement lost power for several decades.²⁰

From the end of the 19th century, the animal model played an important role in vaccinology, first in gaining insight into the etiology and course of disease, second in the development of prevention and, in the case of antisera, of treatment, and finally, in the development of quality control of these preparations. As an example, an overview is given of the animal model in the development of diphtheria antitoxin and vaccine quality control (Table 1.3).

In fact, Koch’s postulates still do apply to today’s vaccine development, and the role of the animal model remains indisputable. However, new technologies have been or are about to be introduced that will modify the role of the animal model. Information will be provided in the following sections, but first statistics and some characteristics of the use of laboratory animals in vaccinology will be presented.

Table 1.2 Animal Models in the Development of Diphtheria Treatment and Prevention

Development	Year	Scientist	Animal Species ^a
Isolation of the causal microorganism <i>Corynebacterium diphtheriae</i>	1884	Loeffler	Pigeon, chicken, rabbit, guinea pig
Production of the exotoxin	1884	Roux and Yersin	Various animal species, guinea pig
Demonstration of the therapeutic value of antitoxin	1890	Behring and Kitasato	Guinea pig , dog, mouse, rat, various animal species
Large-scale production of antitoxin	1894	Roux and Martin	Dog, sheep, goat, cow, horse
Toxin-antitoxin mixtures for active immunization	1913	Behring	Guinea pig
Diphtheria toxoid (vaccine)	1923	Ramon	Various animal species

^aThe animal species finally chosen is in bold type.

Source: Adapted from Hendriksen, C.F.M., in *Replacement, Reduction, and Refinement of Animal Experiments in the Development and Control of Biological Products*, Brown, F., Cussler, K., and Hendriksen, C., Eds., Developments in Biological Standardization 86, Karger, Basel, 1996, 3.

Table 1.3 Animal Models in the Quality Control of Diphtheria Antitoxin and Vaccine

Development	Year	Scientist	Animal Species
Quality control of antitoxin	1892	Behring and Wernicke	Guinea pig
Introduction of a standard preparation in potency testing	1897	Ehrlich	Guinea pig
Potency test using parallel-line bioassay	1937	Prigge	Guinea pig
Multiple intradermal challenge test	1974	Knight	Guinea pig
Serological potency test	1985	Kreeftenberg	Mouse

Source: Adapted from Hendriksen, C.F.M., in *Replacement, Reduction, and Refinement of Animal Experiments in the Development and Control of Biological Products*, Brown, F., Cussler, K., and Hendriksen, C., Eds., Developments in Biological Standardization 86, Karger, Basel, 1996, 3.

CHARACTERISTICS OF THE USE OF LABORATORY ANIMALS IN VACCINOLOGY

Few specific data are available on the use of laboratory animals in vaccinology. In the Netherlands, yearly statistics on the use of animals in biomedical research and testing have included a specific question on biologicals. Figure 1.2, which specifies the purposes of animal use in the Netherlands in 2002, shows that the category of biologicals accounted for 22.7% of total use. Although the use of animals for biologicals has been reduced in the last 20 years, the number has increased as a relative percentage of total use for biomedical research (Figure 1.3). Figure 1.4 further specifies the category of biologicals. Since this information is not included in the official Dutch statistics, the data given are percentages and are based on personal inquiry. As can be seen, vaccine development and vaccine quality control are particularly animal demanding. Although no data are available, it is assumed that the percentage of animal use for biologicals as compared to total animal use will be about the same in other European countries as well as in the U.S. In nonindustrialized countries, the percentage might even be higher as vaccine production and quality control quite often are major areas of biomedical research in those countries. Some other characteristics of the use of animals for the category of biologicals are given in Table 1.4, which shows that many of the animals are required for regulatory purposes — the registration of a new product and routine batch release testing. Furthermore, animal use is also characterized by a high level of pain and suffering. Particular, vaccine potency testing is based on animal models of a general design dating back to Behring and Ehrlich and often includes an immunization–challenge procedure.

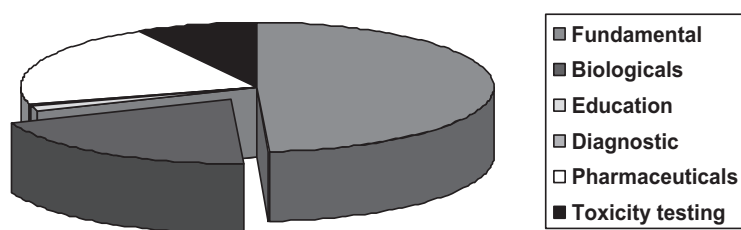


Figure 1.2 Purposes of use of laboratory animals in the Netherlands and percentage of total use. (From Zo doende 1978–2002, Annual statistics on the use of laboratory animals in the Netherlands, Keuringsdienst van Waren (KvW), The Hague, 2002.)

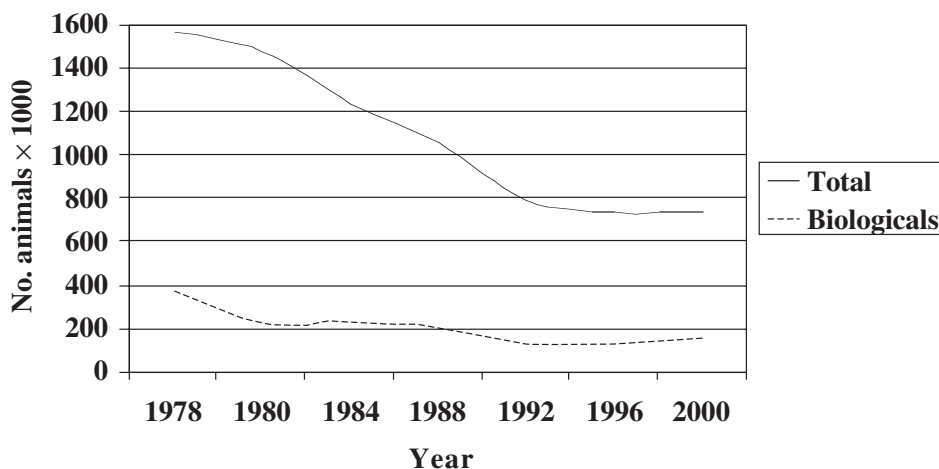


Figure 1.3 Use of laboratory animals in the Netherlands in the period between 1978 and 2002: total use and use for the purpose of biologicals. (From Zo doende 1978–2002, Annual statistics on the use of laboratory animals in the Netherlands, Keuringsdienst van Waren (KvW), The Hague, 2002.)

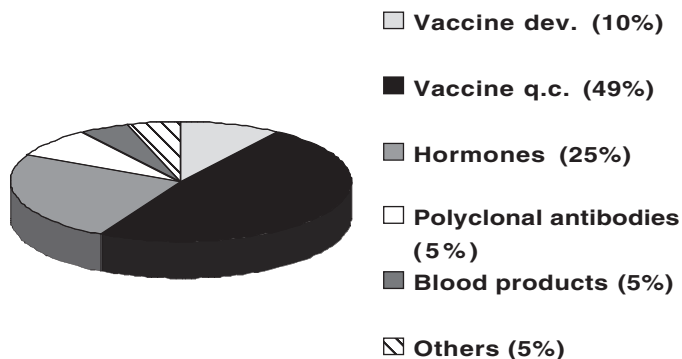


Figure 1.4 Use of laboratory animals for the category of biologicals: specification of purposes. The 0.1% of laboratory animals used for vaccine production is too small to indicate in the diagram. Animals are no longer used for monoclonal production.

Table 1.4 Characteristics of Use of Animals for the Category of Biologicals and for Total Animal Use^a

Characteristic	Percentage for the Category of Biologicals	Percentage for Total Animal Use (All Categories)
Percentage of use of animals	23	100
Use of animals for regulatory purposes	69	28
Substantial pain and suffering	21	13
No pain relief	1	5

^a Biologicals include antisera, immunoglobulins, hormones, vaccines, blood products, cytokines, etc.

Source: Zo doende 2002. Annual statistics on the use of laboratory animals in the Netherlands. Keuringsdienst van Waren (KvW), The Hague, 2002.

ANIMAL MODELS IN VACCINOLOGY

Laboratory animals are still essential in vaccinology for vaccine development, vaccine production, and finally for vaccine batch release. Although *in vitro* methods are used and their contribution to vaccinology is becoming more important, many scientific questions still require an integrated immune system and consequently, an intact animal. A broad range of animal models is used, depending on the type of vaccine, the target animal species, and whether animals are used in development activities, in production, or in quality control. A general outline of these models will be given in the following paragraphs.

Animal Models in Vaccine Development

The first step in vaccine development is the establishment of an infection model to study pathogenesis. Aspects that are dealt with include route of infection, target organs, incubation time, virulence, and clinical disease progress. Well-characterized and relevant infection models are decisive for the success of further studies. For veterinary vaccines, it will be clear that the target animal species is the model of choice, unless there are reasons (e.g., cost or availability) to establish a laboratory animal model. For human vaccines, there is a need for specific laboratory animal models. History shows that when it was difficult to find a suitable animal model, progress stagnated. For instance, this was the case for poliomyelitis vaccine development. Although monkeys could be infected by intracerebral inoculation, for a number of reasons this appeared not to be a particularly suitable experimental animal for this kind of research.

Recent examples of infection models to study vaccine development are those for emerging infections such as HIV and SARS. Several papers have discussed the use of a chimpanzee model for HIV research (e.g., reference 22). However, apart from economical and ethical constraints, the model only partly reproduced clinical progress as seen in humans. Another approach focused on studying animal viruses analogous to HIV, particularly the lentiviruses that induce acquired immunodeficiency syndrome (AIDS)-like illness in animals — SIV (simian immunodeficiency virus) infections in cynomolgus monkeys²³ and FIV (feline immunodeficiency virus) infection in cats.²⁴ Thus information could be obtained about genetic diversity, infection characteristics, etc. More recent is research on SARS. To date, infection models have been described using monkeys (cynomolgus macaques),²⁵ cats, and ferrets.²⁶ Having an infection model also offers a way to upscale virus production or to attenuate the virus by serial passage.

Vaccine development generally starts with obtaining information about immunogenicity. Depending on the type of vaccine, microorganisms will be attenuated/inactivated as is the case for conventional vaccines, or studies will start to identify and select the antigenic structures (epitopes) that are relevant for immunogenicity and could be used as (subunit) vaccine leads. Part of this work will be done *in vitro*, but particularly information on complex immunological processes such as type of immune response (humoral and/or cytotoxic T-cell), antibody classes produced, duration of immune response, protective activity, and antigenic stability often requires the use of laboratory animals. In the case of conjugate vaccines, the effect on immune response due to protein linkage has to be studied, and in the case of vector vaccines, the best vector has to be identified and characterized. Additionally, adjuvants have to be identified that are optimal in enhancing and/or modifying immune responses. Thus, animal studies are performed to study antigen–adjuvant interactions and to select an adjuvant that is potent and safe.

Another focus of study is safety aspects. In the case of conventional vaccines, information has to be provided on loss of virulence, thermal stability, and antigenic stability of attenuated vaccines and on protocols for effective inactivation of inactivated vaccines. In the last few decades safety requirements have increased in diversity, particularly with the introduction of new production technologies. For instance, the safety requirements for registration of rDNA vaccines specify studies on local and systemic toxicity including histopathological effects, virulence of the vector, stability of the integrated sequence, transmission from vaccinated to nonvaccinated animals, reversion of virulence, hypersensitivity, and drug interactions.

Apart from the development of new vaccines, continuing efforts are made to improve existing vaccines, generally to reduce adverse effects, such as is the case for the pertussis vaccine, but also to optimize vaccine efficacy, as for tuberculosis and measles, or to overcome problems of antigenic variation (influenza vaccine).¹⁹ Another area of research is aimed toward the development of combined vaccines. The number of products in pediatric immunization programs has increased significantly in the last few decades and will further increase in the near future. As the acceptance rate for immunization is inversely correlated with the number of injections, there is a need to combine vaccines and to limit the number of injections. Thus, studies focus on the interaction of the various vaccine components as well as on new administration approaches such as prefilled ampules and gene gun “injection.”

Early-phase vaccine development is based on fundamental research. Study protocols, although fulfilling the conditions of Good Laboratory Practice (GLP) and Good Manufacturing Practice (GMP), are not laid down in formal procedures. However, toward the end of development, studies, in particular those on immunogenicity and safety, become part of the registration dossier for obtaining the product license. Registration dossiers for product licenses are approved in Europe by the European Agency for the Evaluation of Medicinal Products (EMA) and in the U.S. by the Food and Drug Administration (FDA) or the U.S. Department of Agriculture (USDA). Generally, registration is indicative of the animal models that will be specified for batch release testing.

Animal Models in Vaccine Production

Laboratory animal use in vaccine production is limited in numbers. Bacterial microorganisms are grown in culture media that might require blood products or beef extract. Due to the BSE (bovine spongiform encephalopathy) crisis, however, there now is a strong tendency to use synthetic media only, without any animal products. Virus production needs a cell substrate for propagation. Until the introduction of technologies that allowed for large-scale cell culture, viruses had to be grown in living animals (rodents or nonhuman primates) or in embryonated eggs. For example, the rabies vaccine was produced in brains of suckling mice until cell culture–produced vaccines became available in the seventies.²⁷ Now, rabies vaccines are generally produced in cell culture, but *in vivo* production is still used, particularly in developing countries, because these vaccines are cheap and easy to produce, despite the safety disadvantages of animal-produced vaccines. Only a few viral vaccines are still based on *ex vivo* production. One of these is the polio vaccine. Some manufacturers still rely on virus propagation in primary or subcultured monkey kidney cells, although the virus can also be produced in continuous cell lines (CCLs) such as Vero cells or human diploid cells. Table 1.6 presents an overview of cell culture and non–cell culture virus vaccines.

Apart from the greater safety of vaccines produced in CCLs, the use of CCL in the manufacture of viral vaccines has other advantages:

1. The cell culture substrate is more consistent and “clean” than primary culture.
2. The use of large-scale tissue culture procedures allows more efficient and more reproducible cell growth.
3. It provides higher yields of virus.
4. It could reduce or even preclude the use of animals.²⁹

Despite these advantages, the acceptability of CCL as a substrate in the production of viral vaccines has been controversial due to the suspected oncogenic potential of CCLs — that is, the potential to induce cancer in humans after vaccine administration. However, it has now been well established that DNA obtained from these cells has no detectable activity *in vivo*.³⁰ The use of primary cell cultures has continued since then because vaccine safety is not only a scientific issue but a political issue as well. However, there is reason to believe that virus propagation in CCLs will gradually but completely replace the use of animals.

Table 1.6 Cell Culture and Non–Cell Culture Virus Vaccines

Cell Culture Virus Vaccines	Non–Cell Culture Virus Vaccines
Polio	Smallpox (vaccinia): bovine, lymph, skin
Measles	Rabies: various brain or spinal cords (rabbit, sheep, goat, mouse), duck embryo
Mumps	Japanese encephalitis: mouse brain
Rubella	Yellow fever: mouse brain, hen's egg
Adenovirus	Influenza: chick embryo allantoic fluid
Rabies	
Varicella	
Hepatitis A	
Rotavirus	
Cytomegalovirus	

Source: Katz, S.L., Wilfert, C.M., and Robbins, F.C., in *Vaccinia, Vaccination, Vaccinology. Jenner, Pasteur and Their Successors*, Plotkin, S.A. and Fantini, B., Eds, Elsevier, Paris, 1986, 213.

Animal Models in Vaccine Quality Control

Central to the vaccine batch release process is quality control. In particular, conventionally produced vaccines have a tight testing program because these products are complex mixtures that might differ from batch to batch. New-generation vaccines can be produced more consistently, and consequently quality control, and thus the use of laboratory animals, is less extensive. Quality control takes place within a regulatory framework, and guidelines for quality control tests are laid down by (inter)national regulatory bodies such as FDA and USDA for the U.S. and the European Pharmacopoeia for the Member States of the Council of Europe. Broadly, controls are directed almost entirely towards the safety or the lack of toxicity and the efficacy or potency of the vaccine. Control on safety ensures that the vaccine does not contain ingredients that are harmful to humans or animals after administration. The harm can be traceable to the agent (the bacterial or viral strain or toxin), to chemicals added (intentionally or unintentionally), or to the substrate used (culture media, culture cells, or embryonated hen's eggs). Table 1.7 specifies the tests that, depending on the type of vaccine, can be part of the safety-testing scheme.

Next to safety testing, by far the greatest effort in quality control goes into potency testing to demonstrate that the vaccine induces protective immunity after its administration. In the case of live vaccines (e.g., mumps, oral polio, bacille Calmette-Guérin [BCG]), the efficacy of each vaccine batch is related to the number of live particles, determined either by counting or by titration, that is, entirely *in vitro*. Potency testing of inactivated vaccines, however, generally requires the use of experimental animals. The tests often rely on a limited number of basic principles, quite often relating to procedures already established in the early days of vaccinology. One of the approaches (the "parallel-line" potency test) is to immunize groups of animals with serial dilutions (three or four) of the vaccine under study and a reference preparation with known potency, respectively. Generally, these ranges of dilutions include groups of animals receiving a low, an intermediate, and a high vaccine dose. After a number of weeks, the animals are challenged with the virulent microorganism or toxin, and specific clinical signs or death is recorded for the observation period given. Based on the information obtained from the various groups, a dose-response curve can be plotted, both for the vaccine under study and for the reference preparation, and the dose that protects

Table 1.7 Safety Tests in Quality Control of Vaccines and Animal Models Used

Safety Aspect	Specific Safety Test	Animal Model Used
The agent	Specific toxicity test (bacterial vaccines) ^a	Mice, guinea pigs
	Identity test (all)	
	(Neuro)virulence test (live vaccines) ^a	Monkeys (intracerebral and intraspinal)
	Test for residual live virus ^a (live vaccines)	Various animal species
Vaccine additives	Test for endotoxin ^a	Rabbits
	Abnormal toxicity (= innocuity) test ^a (all)	Mice and guinea pigs
	Target animal safety test (veterinary vaccines) ^a	Target animals
	Thimersal content	
	Sterility test (all)	
	Test for endotoxin levels ^a	Rabbits
	Chemical tests to characterize components	
Substrate used	pH (all)	
	Tests for extraneous microorganisms* (live)	Various animal species, e.g., suckling mice, chickens
	Test for tumorigenicity	Mice

^a Tests that usually are based on animal models.

Source: Adapted from Hendriksen, C.F.M., *Laboratory Animals in Vaccine Production and Control. Replacement, Reduction, and Refinement*, Kluwer Academic, Dordrecht, the Netherlands, 1988.

50% of the animals is calculated. An alternative strategy for toxoid vaccines is to immunize one group of animals with the vaccine under study, to bleed these animals after a number of weeks, and to estimate levels of protective antibodies by titration of serum samples mixed with fixed doses of toxin in groups of animals. Both approaches require large numbers of animals (e.g., 140 for the parallel-line potency test) and induce substantial levels of suffering.

Attention is now being given to the development of methods that could replace, reduce, and refine (the 3Rs) the use of laboratory animals. The reasons for this trend include:

- Concern about the extensive use and the substantial levels of pain and distress inflicted on the animals
- The questionable relevance of some animal models, such as the rabies vaccine and whole cell pertussis vaccine potency tests, which show very poor reproducibility
- The fact that animal tests are time consuming and interfere with the limited shelf life of vaccines
- New developments and strategies in vaccine production such as standardization of production processes and the introduction of GMP, Quality Assurance (QA), and in-process control that make extensive quality control less relevant and even superfluous.

Extensive overviews of 3R developments have been published.^{31–33} A summary of some of the achievements is given in Table 1.8.

Although these developments had an impact on the numbers of animals used, a breakthrough can only take place if the concept of “demonstration of consistency” is generally accepted. The key issue of consistency has emerged from the new generation of vaccines. These vaccines, which are based on new technologies, are produced in a consistent way and the stress of quality control is on in-process monitoring rather than on final batch testing. In-process testing is almost exclusively based on *in vitro* biochemical and physicochemical tests. The consistency concept has become state of the art for the new generations of vaccines. Also, in the field of conventional vaccines, continued advances in production technology have resulted in more defined and thus less variable products. This, together with the implementation of GMP and QA, makes people feel that for a conventionally produced vaccine, the extent of batch release testing should reflect the level of consistency obtained with the vaccine. Thus, a vaccine manufacturer should perform extensive testing (including animal testing) during the development phase and on the first few batches of the new product to characterize the vaccine thoroughly. However, if consistency in production is demonstrated, then testing could rely on a battery of easy-to-use *in vitro* assays to characterize (fingerprint) the vaccine and confirm consistency. If this new approach is applied, the number of animals used for quality control of conventional vaccines will be reduced to an absolute minimum.

Table 1.8 Summary of Major 3R Developments in Vaccine Quality Control

Vaccine	Animal Test	3R Alternative
Toxoids	Potency test based on challenge procedure	Serological-based potency test
Erysipelas	Potency test based on challenge procedure	Serological-based potency test
All	Abnormal toxicity test	Deleted from test specifications ^a
Hepatitis B	Mouse potency test	<i>In vitro</i> method (ELISA)
Polio (live)	Neurovirulence test in monkeys	MAPREC assay and transgenic mouse test
Relevant vaccines	Lethal challenge procedure	Use of humane endpoints

Note: ELISA = enzyme-linked immunosorbent assay; MAPREC = mutant analysis by polymerase chain reaction (PCR) and restriction enzyme cleavage.

^a Deleted only from the European Pharmacopoeia monographs but still specified by other regulatory bodies.

CONCLUSION

Animal models have played and still play an essential role in vaccinology. They have made possible the worldwide immunization of children with pediatric vaccines that are both safe and efficacious. Until recently, laboratory animals were required for development, production (in case of viral vaccines), and particularly, for quality control. This resulted in the use of large numbers of animals for these purposes, often in models that induced severe pain and suffering. Changes are now taking place that will affect this situation. Newer generations of vaccines are more defined and can be produced more consistently than the conventionally produced vaccines were. As a consequence, a shift in the need for laboratory animals will take place. The burden of animal research with the newer generation of vaccines will be on development, while for the conventional vaccines it was on routine batch quality control. Therefore, it can be anticipated that the numbers of animals needed will be reduced in the near future. However, limited numbers will still be needed to evaluate the interaction of the vaccine with the complex immune system in an intact organism.

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CHAPTER 2

Animal Models for Human Behavior

Steven J. Schapiro

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INTRODUCTION

This chapter will provide a very brief treatment of a very broad topic — animal models for human behavior. Although humans tend to think of themselves as unique, and a few human behaviors are difficult to model using animals, many human behaviors can be effectively studied using animal models. From learning to avoid a shock¹ to learning to use a language,² from suffering from the effects of social deprivation³ to benefiting from the effects of environmental enrichment,⁴ studies of animal behavior have taught us a great deal about human (and animal) behavior. Most behavioral studies of laboratory animals have as their goal the exploration, explanation, and/or prediction of human behavior. Flatworms, fruit flies, sea slugs, pigeons, parrots, mice, rats, dogs, monkeys, dolphins, and chimpanzees are all organisms whose behavior has been investigated in the laboratory in an attempt to model, to simulate, or to understand human behavior. This chapter

will focus on studies that have taken place in the laboratory setting but will also include occasional mention of work that has made use of animals in the natural setting (the “field”) as models for human behavior. Additionally, the chapter will emphasize nonhuman primate models for human behavior, as these species are perhaps the most relevant for behavioral work. However, where appropriate, studies of nonprimate models will also be discussed. The research cited in this chapter includes behavioral data collected from at least 38 different animal taxa.

There are numerous motivations for studying animal behavior as a means of increasing our understanding of human behavior. Behavioral processes seem to lend themselves to evolutionary explanations more easily than physiological or disease processes do, so animal models for human behavior seem to regularly invoke such evolutionary explanations. Perhaps the clearest reason for using animals as behavioral models is that certain experimental manipulations that may not be possible or ethical to perform with humans can be performed with animals. Although retrospective or epidemiological-type analyses can contribute greatly to our understanding of human behavior, prospective, controlled experimental investigations can tell us considerably more. Thus, the behavioral effects of an experimentally induced lesion to the hippocampus of a mouse⁵ or the effects of glucocorticoids on the hippocampus in aged primate brains⁶ can be studied to learn more about the behavioral effects of Alzheimer’s disease in humans.

Behavioral data, as well as physiological and molecular data, contribute to our understanding of human conditions. This is true (both directly and indirectly) in a number of different contexts. For instance, even in studies where the focus of the experimental hypotheses is not directly on behavioral measures, understanding the normal behavioral requirements of the animal model and effectively managing the behavior, needs, and care of the animal can aid in the creation of a valid, well-defined animal model. Clearly, the better defined animal models are, the more directly investigators will be able to test their experimental hypotheses, minimizing the influence of confounding variables and maximizing reliability and validity.

One of the most effective uses of behavioral assays in laboratory research is in understanding the normal behavior patterns of an animal model and then measuring deviations from these normal patterns as a function of an experimental manipulation. This can be accomplished simply by systematically observing the animals’ behavior and/or by training the animal to perform specific tasks. An animal that has been trained to successfully avoid shock under normal conditions but that fails to avoid shock while under the influence of alcohol⁷ provides a useful model for alterations in human behavior as a function of alcohol consumption.

This chapter is organized into several major sections, focusing on the development of behavior; social behavior; cognitive behavior; nonsocial behavior; and abnormal behavior. Since chapters in Vol. II of this series have focused on animal models in pharmacology,⁸ in neuroscience,⁹ and for psychological disorders,¹⁰ only limited attention will be devoted to these topics in the present chapter. Table 2.1 summarizes, in tabular form, most of the information contained in the sections that follow.

DEVELOPMENT OF BEHAVIOR

Perhaps the best place to begin is with a very brief discussion of how behavior develops. While the relative influence of genetic and environmental factors on behavioral outcomes is still very much under debate, it is clear that behavior can be influenced (positively or negatively) by social and environmental circumstances during critical developmental periods.¹¹ In concise terms, deprivation during specific developmental periods usually results in decrements in behavior,³ and enrichment during specific developmental periods usually results in improvements in behavior.¹¹ Rats and nonhuman primates have served as the most illustrative models for studying the development of behavior, and this work has provided critical insights into factors that affect the development of both normal and abnormal human behavior.

Table 2.1 Selected Animal Models for Human Behavior

Behavior	Relevant Animal Models	Representative References
Development of behavior	Rats, rhesus	3, 4, 16
Social deprivation	Rhesus	3, 12
Enrichment and handling	Rats	4, 11, 17–19
Social behavior	Many	
Communication	Rhesus, chimpanzees	24, 25
Language	Parrots, dolphins, honeybees, chimpanzees	2, 26–31
Culture	Chimpanzees	39
Affiliative behavior	Cynomolgus monkeys	40
Conflict resolution	Rhesus, chimpanzees	42, 43
Sexual behavior	Rats, rhesus, bonobos	44–48
Play behavior	Hamsters, rhesus	50, 51
Caregiving behavior	Rhesus	53, 78
Parental	Rhesus, vervets	13, 14, 57
Alloparental	Jays, ibex, marmosets	59, 60, 62, 63
Adoption	Baboons, penguins	66, 67
Aggressive behavior	Ants, rats, lions, chimpanzees	69–71, 74, 77
Dominance	Baboons	86
Subordination	Baboons	85
Infanticide	Capybara, lions, langurs	74–76
Cognitive behavior	Pigs, capuchins, bonnet monkeys, baboons, chimpanzees	81–84, 91–93
Learning and memory	Gerbils, dogs, sea lions, dolphins, nutcrackers	1, 87, 99, 101, 102
Trained to “criterion”	Pigeons, rhesus, baboons	105, 110, 118
Counting	Parrots, chimpanzees	107–109
Tool use	Capuchins, oranges, chimpanzees	106
Nonsocial behavior		
Sleeping	Rats	111
Eating	Rats, rhesus	114, 115
Manipulation	Capuchins	113
Abnormal behavior	Rats, rhesus	116
Stress	Rats, dogs, vervets, baboons	85–89
Psychopathology	Rhesus macaques	3, 10, 12
Addictive behavior	Baboons	118
Lateralized behavior	Chicks, marmosets, chimpanzees	1, 87, 99, 101, 102

As Murison¹⁰ has pointed out in his chapter in Vol. II of this series, the work done by Harlow, his colleagues, and their students has clearly revealed the dramatic negative long-term behavioral and physiological consequences of social deprivation (primarily maternal separation) during early development for rhesus monkeys.¹² Many human applications of this work exist, and in fact, the human and nonhuman literatures on this topic are quite intertwined.¹³ Significant changes to human parenting practices have occurred as a result of the lessons learned from the work done with infant macaques.¹⁴ Attempts to establish secure attachments for human infants by increasing “contact comfort,”^{3,12} even among infants recently adopted from orphanages, may be one of the clearest examples of such changes.¹⁵ Rhesus macaques were particularly good models for this type of work (which could not be performed on humans), far superior to the more altricial, and thus fragile, rodent models to be discussed next.

Not only has work with animal models identified the negative consequences of early social deprivation for animals and humans, it has also revealed the positive consequences of early enrichment. Rats exposed to an enriched laboratory environment or to handling (to be distinguished from maternal separation) early in life have been shown to demonstrate numerous behavioral and physiological advantages over animals that were raised in a “normal” laboratory environment or that were not handled.¹⁶ Even individual differences in the amount of time that mothers spend grooming their young have been shown to have long-term behavioral consequences.¹⁷ Although the question arises as to whether the normal laboratory environment can really be considered normal (rather than impoverished¹⁸), the data still support the notion that additional stimulation during development results in beneficial changes.¹⁹ These data have clearly been applied to the human situation, as numerous strategies are now available to enrich the prenatal and postnatal environments during various stages of development (e.g., playing music,²⁰ teaching gestures²¹).

SOCIAL BEHAVIOR

Human social behavior is perhaps the most complex array of behaviors one can imagine. While few species demonstrate as complete a constellation of complex social behaviors as humans do, many animal species display subsets of this array and can thus serve as models for certain human social behaviors. One of the more interesting aspects of human social behavior is social cognition, the cognitive processes that help shape social interactions.²² Clearly, our social behaviors are based not only on our assessments of the results of past interactions but also on our predictions of the potential results of future interactions. Similarly, humans regularly assess the social implications of their behavior, attempting to take the viewpoints of other members of their “group.” Similar processes seem to apply to, and can be studied in, some primate species;²³ these social cognition abilities will be discussed below and again in the section on cognitive behaviors.

Communication

Clearly, the ability of humans to effectively communicate with one another is one of the most impressive social behaviors of any animal species. The debate rages over whether or not animals are capable of using language, but the relevant point for this chapter is that several useful models exist for studying language abilities in animals.² Although language is the most glamorous of the types of communication that are under investigation in animals, other channels of human communication can certainly be studied using animal models as well. Studies of gesturing in chimpanzees²⁴ and “body language” in macaques²⁵ are two modalities that come immediately to mind.

A variety of animal species have been involved in laboratory investigations of the evolutionary origins of language. Clearly, the sign language and lexigram studies with great apes² are the most familiar studies of animal language to the average person, but African grey parrots,²⁶ several Old World monkey species,^{27,28} dolphins,²⁹ and honeybees³⁰ also figure prominently in investigations of language. Language research, particularly studies involving vervet monkeys²⁷ and honeybees,³¹ is particularly well suited for field experiments, where the “lab” is the natural setting, independent variables (vocalizations, location of food sources) can be readily manipulated and controlled, and the behavior of the animal model observed. The fact that these studies take place in the natural setting adds considerable validity to these models for understanding human language. Early studies of animal language were subjected to considerable methodological criticism,³² so more recent studies of nonhuman language³³ have implemented methodological enhancements designed to limit potential criticism and to increase validity.

Culture

Another of the hallmarks of human behavior is the ability to display culture. Culture can be described as “a pattern of behaviors (or their material manifestations or informational content) that is socially transmitted, rather than genetically inherited or stimulated by particular environmental conditions.”³⁴, p. 57 Forms of communication³⁵ and “good manners” would be two examples of behavioral traditions that exemplify culture in humans. Just as the debate rages concerning whether studies of animal language are relevant to the study of the origins of human language, there is considerable controversy over whether animals are capable of culture.³⁶ While several experimental studies in the laboratory have shown that animals can display some of the behaviors and tendencies potentially indicative of culture,³⁷ not all investigators accept this interpretation.³⁸ There appears to be considerable resistance to the idea that animals could display culture and therefore serve as models for investigations of the origins of human culture. So once again, the methodologies of the investigations that have supported the idea that animals can display culture have been criticized.³⁶ Perhaps the most convincing data in support of animal models for human culture come from long-term field studies of chimpanzees that reveal that some populations of chimpanzees display par-

ticular behaviors/practices/traditions and that other populations, living under similar ecological conditions, do not.³⁹

Affiliative Behavior

Two examples related to the study of affiliative behavior in animals have great relevance to the study of human behavior. The first involves the effects of affiliation and social support on physiological (immunological) responses and function.⁴⁰ The data from both humans and nonhuman primates suggest that high levels of affiliative behavior and social support have positive physiological (immunological) consequences. The potential for adapting findings from nonhuman primate models to human behavioral therapies aimed at enhancing immune responses is great and has been at least partially realized.⁴¹ While retrospective studies of the relationship between affiliative behavior and immune function are possible in both human and nonhuman subjects, prospective studies that experimentally isolate the effects of specific manipulations may be easier to conduct using nonhuman subjects.

A second relevant example of animal affiliative behavior modeling human behavior is conflict resolution. Individuals of many species of nonhuman primates reconcile with one another after an aggressive encounter.⁴² This can take the form of either the aggressor reconciling with the recipient of the aggression or vice versa. Reconciliation is a complex and potentially dangerous social behavior that puts one or both animals at risk of additional aggression. However, the advantages of maintaining a cohesive social group are thought to outweigh the risks of additional aggression in these scenarios.⁴³ As one can easily imagine, the study of animal conflict resolution provides crucial insights into the evolution of techniques for maintaining cohesion in human social groups.⁴³ Even so, relatively little is known about how humans resolve conflicts, and especially when and why humans apologize.

Sexual Behavior

The study of sexual behavior in animals has contributed greatly to the understanding of sexual behavior in humans, particularly in relation to the effects of hormones on sexual behavior.⁴⁴ This has considerable relevance to the general topic of birth control. Studies of rats in the laboratory have identified loci in the brain associated with sexual activity,⁴⁵ and more current investigations are assessing the effects of sexual practices on sexually transmitted diseases and the efficacy of related vaccines.⁴⁶

Ovulation is concealed in humans, and this fact has important implications for human sexual behavior and for paternal behavior. Human males may be more likely than males of some other species to invest in infants, since they cannot be completely certain that they sired any particular infant, and human females may attempt to take advantage of this. As with some of the other behaviors previously discussed, the sexual behavior of animals can serve as an explanatory model for the evolution of human sexual behavior. Bonobos are a particularly important model in this regard, since unlike most animal species in which sexual behavior is under "hormonal control," bonobo (and human) sexual behavior appears to be less constrained by hormonal influences.⁴⁷ Even more importantly, bonobos use sexual behavior for nonreproductive purposes,⁴⁸ as a critical component of their social behavior repertoire, a situation that is more typical of humans than of other animal species.

Play Behavior

Humans, particularly infants, children, and other youngsters, typically spend a fair bit of their time engaged in play. Although we all could probably identify humans or animals playing, it is somewhat more difficult to provide a precise scientific definition of what constitutes play. One

reasonable description states that “play incorporates many physical components of adult behavior patterns,... but without their immediate functional consequences. Play is also more exaggerated, repetitious, and variable than corresponding nonplay behavior.”⁴⁹ Animals (again, especially youngsters) also spend much of their time engaged in playful activities; however, for animals, an evolutionarily based justification for the play behavior is typically identified to explain the behavior.⁵⁰ This explanation is typically related to the practicing (during play) of some behavior that will be critical later in life. For example, young male hamsters engage in play fights with one another in order to practice fighting skills to be used to attain mating opportunities in adulthood.⁵¹ Current investigations of play behavior in animals have focused on the assessment of play as a behavioral assay to measure deviations from the normal condition or on the use of play as therapy in studies of models of psychopathology (e.g., attention deficit hyperactivity disorder [ADHD]).⁵²

Caregiving Behavior

Humans typically take very good care of their offspring, investing great quantities of time and resources in their young. While parents of teenagers today may feel that their investment is occasionally excessive, there are multiple examples of extreme caregiving behavior in the animal world as well.⁵³ And as with most of the topics in this chapter, much has been learned about human caregiving from studies of animal caregiving. Typically, when the behavior of animals is explained, evolutionary justifications for behavioral patterns are provided. Although evolutionary explanations no doubt fit certain patterns of human behavior, there seems to be considerably more flexibility when attempting to explain human behavior. For example, although male primates may kill infants (infanticide) sired by rival males to increase their own likelihood of siring infants, this type of explanation is unlikely to be invoked in human situations.

Many of the evolutionary constraints that influence the parenting behavior of animals also influence human parenting behavior. Offspring of altricial species (such as humans) typically have long developmental periods requiring considerable care from one or both parents,⁵⁴ so the most representative models for human parenting are other altricial species (some birds, rodents, primates). Mothers and fathers differ considerably in their initial investment in offspring, and much has been made of this discrepancy as an explanation for differences in parenting behavior.⁵⁵ In general, a positive correlation exists between a male’s certainty of paternity and his tendency to engage in paternal behavior (for both humans and animals).⁵⁶ As one example, males of species with typically monogamous mating systems (marmosets), on average, are considerably more paternal than males of species with typically polygamous mating systems (macaques).⁵⁷ Within and across human cultures, some males behave like marmosets and others behave like macaques.

Human parents often rely on caregivers other than themselves to assist in the raising of offspring. There are numerous examples of similar alloparenting situations in the animal world. Ungulate mothers may leave their infants in “day care” with a “babysitter” while they go out to forage.⁵⁸ Older siblings help care for younger siblings in Florida scrub jays,⁵⁹ several marmoset species,⁶⁰ and humans.⁶¹ Aunts and cousins may be regular alloparents in many species of nonhuman primates^{62,63} and in humans.⁶⁴ Although grandparents are frequent alternate caregivers among humans,⁶⁵ this is considerably less common among animals (but see Reference 57). There are even examples of individuals purposely “adopting” and raising unrelated orphans (at significant costs to themselves and/or their own offspring) in several species.^{66,67}

Aggressive Behavior

One of the most puzzling aspects of human behavior is the amazing array of aggressive, violent, and destructive behaviors of which we are capable. One look at the newspaper will provide innumerable examples of humans’ inhumanity toward other humans. But should this really be that surprising? Do examples of similar aggressive/violent tendencies exist in the animal world? Based

on data from both the laboratory and the natural setting, the answer is, of course, yes. Natural selection⁶⁸ suggests that animals are constantly competing with one another to garner sufficient resources to make certain that their genes are passed on to future generations. Although this competition is often aggressive and/or violent, it is less frequently lethal, and according to evolutionarily based arguments, it is always focused on competition for resources⁶⁸ and is therefore rarely performed for “no apparent reason.” On the other hand, ants and chimpanzees engage in war;^{69,70} chimpanzees engage in cannibalism;⁷¹ orangutan males rape orangutan females;⁷² male zebras,⁷³ lions,⁷⁴ langurs,⁷⁵ and rodents⁷⁶ commit infanticide; rats kill mice;⁷⁷ and under certain circumstances, mother macaques become abusive toward their infants.⁷⁸ Clearly, war, cannibalism, rape, infanticide, and child abuse are all “human” behaviors.

Additionally, a variety of brain stimulation/lesion studies in humans and animals have identified anatomical structures in the brain that are associated with the expression of aggressive behavior. For instance, stimulation of the medial hypothalamus, amygdala, and connected pathways results in aggressive behaviors,⁷⁹ as does lesioning of the ventral striatum, septal area, and adjacent structures.⁸⁰

While we rarely discuss agonistic (dominance and subordination) interactions among humans in the same terms that we use for discussions of agonistic interactions among animals, this neither means that humans do not engage in agonistic interactions nor that animals would not be useful models for human agonistic interactions. In fact, several aspects of animal agonistic interactions are of great relevance to the human condition. Perhaps the most interesting is the social awareness/cognition/theory of mind that is involved in being able to assess dominance and subordination within a social group. Not only must humans and animals be able to assess and remember their own position and alliances within a dominance hierarchy, but they must also be able to reasonably accurately assess and remember the positions and alliances of all of their groupmates.⁸¹ Additionally, they must constantly evaluate the costs and benefits of specific behaviors based not only on their knowledge of previous interactions, but also on their predictions of what would transpire in future interactions.⁸² Clearly, this is what people do on a regular basis and may sound quite sophisticated on a surface level. However, it appears that many animal species other than primates are able to perform similar social evaluations.⁸³ Coalition formation, on the other hand, is fairly sophisticated and seems to be more prevalent among species considered to be more socially “advanced.”⁸⁴

The effect that chronic subordination has on the physiological systems of low-ranking baboons is another relevant animal model for human agonistic behavior. To make a long story short, the chronically high levels of glucocorticoids observed in chronically stressed, low-ranking male baboons are frequently associated with a variety of physiological impairments, including most importantly, death of neurons in the hippocampus.⁸⁵ Baboons, like humans, experience chronic stress accompanied by glucocorticoid secretion and therefore may suffer severe physiological consequences. Other organisms, such as zebras, seem to be neither chronically stressed nor do they appear to suffer the consequences of excessive glucocorticoid secretion.⁸⁶ As mentioned previously, there are a number of animal models for inducing stress and for measuring the behavioral and physiological consequences of stress (learned helplessness in dogs,⁸⁷ water maze stressors in mice/rats,⁸⁸ stomach lesions in recently captured vervet monkeys⁸⁹).

COGNITIVE BEHAVIOR

There are a number of “cognitive” behaviors for which animal models can provide considerable information concerning the human condition. Social cognition, as it applied to social hierarchies, has been mentioned previously. This section will focus on two specific topics: (1) other types of social cognition and (2) learning and memory. Although at some levels, those studying these types of behaviors in animals are interested in the phenomena as they apply to animals, they are also interested in the insights into human cognition that can result from the animal data. In fact, from

granting and/or publishing perspectives it is often critical to be able to relate cognitive phenomena in animals to similar or related abilities in humans. Although some interest exists in exploring whether cuttlefish learn through sign tracking or goal tracking,⁹⁰ the real importance of this type of research is to better understand human learning.

Two relatively recent avenues of inquiry into animal social cognition provide some very interesting data. The first is an exploration of “theory of mind”⁹¹ in chimpanzees. These studies are designed to essentially answer the question: can one chimpanzee take the viewpoint of another chimpanzee? In some of these types of investigations, chimpanzee A can see a threat to chimpanzee B that chimpanzee B cannot see. The stimulus is only a threat to chimpanzee B (not to chimpanzee A) and the goal is to see whether chimpanzee A will “warn” chimpanzee B. In general, the chimpanzee that can see the threat will warn the animal that cannot see the threat, providing some data that suggest that chimpanzees could have a theory of mind.⁹² A second example of social cognition involves animals demonstrating an understanding of fairness.⁹³ In these studies, the reactions of one capuchin monkey are assessed when another monkey receives a reward that the experimenters have determined should be perceived as “unfair” by both monkeys. Interestingly, the monkey victimized by the unfairness displays behavioral reactions quite similar in appearance to human indignation, suggesting that these animals possess a concept of fairness.⁹³

A plethora of animal species have served as subjects in psychologists’ investigations of learning and memory, providing ideal models for experimental manipulations of the myriad parameters that influence how humans and animals learn and remember. While learning and memory can definitely be studied in humans, animal models often provide more tightly controllable experimental systems, where independent variables can more easily be isolated from potential confounds.⁹⁴ Of course, findings from learning and memory studies using human subjects have considerably greater validity than those from animal studies. Although white rats and pigeons, and Pavlovian conditioning and Skinnerian conditioning, are the “rules” in studies of learning, many other types of learning phenomena have been studied, and many other animal models utilized, including fruit flies,⁹⁵ sea slugs,⁹⁶ cuttlefish,⁹⁰ turtles,⁹⁷ Japanese quail,⁹⁸ Clark’s nutcrackers,⁹⁹ gerbils,¹⁰⁰ sea lions,¹⁰¹ dolphins,¹⁰² rhesus monkeys,¹⁰³ and chimpanzees.¹⁰⁴ The range of experimental apparatuses available to make these assessments is equally diverse, extending to tasks that require the subjects to utilize computerized joysticks or touch screens (that have been made “animal-proof”)¹⁰⁵ or make and/or use tools.¹⁰⁶

African grey parrots and chimpanzees appear to be able to demonstrate to us that they can count,^{107–109} a talent that some find amazing. It would not be surprising if many species could “count,” however, since a prey animal (zebra) that was able to tell that only six of the eight predators (lions in the pride) were visible would have a selective advantage over those prey animals that could not. It is quite interesting to note all the things that animals are capable of learning and remembering and to understand some of the ways that animal models and experimental systems have been developed to provide usable data. Recent analyses of animal learning have emphasized methods that take advantage of the ecological contexts within which these behaviors typically occur, thereby enhancing validity and our ability to understand human learning processes.

One of the most profitable applications of animal learning models is as a behavioral assay for measuring the effects of experimental manipulations. The easiest example to visualize is training an animal on a task until it is able to reliably satisfy specific performance criteria. Once the animal can reliably achieve these performance criteria, it is then a simple matter to perform experimental manipulations and measure changes in performance as a function of the manipulation. These techniques are particularly useful in studies of drug, brain lesion, and stress effects, where an animal “under the influence” of the experimental manipulation may perform significantly more slowly or less accurately than it did under baseline or control conditions.¹⁰⁵ The ability of many species to learn to perform a variety of simple and complex target tasks reliably, consistently, and at high rates during baseline/control conditions¹¹⁰ emphasizes the value of this type of model.

NONSOCIAL BEHAVIOR

In addition to serving as useful models for sophisticated human social behaviors and cognitive behaviors, animals can also be valuable models for relatively simple, nonsocial behaviors. Sleeping,¹¹¹ feeding,¹¹² and exploration/manipulation¹¹³ are three simple nonsocial behaviors that immediately come to mind. Insights into the behavioral mechanisms related to feeding are of critical importance, given the epidemic of obesity that is currently confronting at least the United States. Any data that would help enable understanding of the behavioral factors that impact eating behavior and obesity, or could aid in the design of strategies that might reduce obesity, would be of great value. Considerable data are available that demonstrate the lifespan and health benefits of caloric restriction,¹¹⁴ but few of these studies address behavioral issues. Similarly, good data are available (from animal models) that have identified brain centers, molecular pathways, and genetic loci that are involved in the development of obesity;¹¹⁵ however, these studies are more likely to identify the underlying causes of eating behavior than they are to study eating behavior as a phenomenon.

ABNORMAL BEHAVIOR

Numerous animal models exist for behaviors that can be considered abnormal in humans. In most of these models, animals are subjected to atypical environmental conditions that result in the animals displaying abnormal behavior patterns. For instance, monkeys engage in abnormally high levels of self-directed grooming when housed alone,¹¹⁶ dogs develop learned helplessness when subjected to inescapable shock,⁸⁷ and mice display increased aggression when living in crowded conditions.¹¹⁷ Animal models for psychopathology have been discussed in Vol. II of this series,¹⁰ so we will just state that some animal models, specifically primate models, have helped shed light on human behavioral abnormalities. The social deprivation studies of Harlow and his colleagues are the clearest example.^{3,12}

Addictive behavior of humans is one type of abnormal behavior that can be effectively modeled using animals and could probably not be prospectively tested using human subjects. Drug self-administration studies using baboons and other species have clearly demonstrated the highly reinforcing nature of some drugs and can help explain some of the abnormal behavior observed in humans addicted to drugs.¹¹⁸ In addition to helping understand how addictive behavior can develop, these studies can also provide insight into potential strategies to rehabilitate those suffering from an addiction.¹¹⁹

LATERALIZED BEHAVIOR

Prior to concluding this chapter, it will be valuable to include one category of behavior that is probably studied *only* as a model for human behavior. Humans display lateralization of behavior (handedness is the clearest example), as do many animal species.¹²⁰ Although there may be some interest in animal lateralization for its own sake, the primary focus of this type of research is to provide insight into the hemispheric specializations that are typical in human brains.¹²¹ While it is extremely likely that there are practical implications of understanding the evolution of caregiving, affiliative, aggressive, or cognitive behaviors in animals, there are far fewer practical implications of understanding the expression of lateralization in chicks,¹²⁰ marmosets,¹²² and chimpanzees.¹²³

CONCLUSION

This has been a brief and selective review of some of the research that is available on the behavior of animals as models for human behavior. The references in this chapter contain data from at least 38 different nonhuman taxa, and an attempt has been made to provide references that are among the most original, most illustrative, and/or most recent for the points being discussed (see Table 2.1). An exhaustive list of citations and species would be neither possible nor useful. Similarly, a complete treatment of all human behavioral factors that could be modeled in animals is probably not possible in a short chapter. For instance, animal “personality” and “emotions” are currently topics of considerable interest,^{124–125} yet it is difficult to figure out how to include them in a chapter focusing on models for human *behavior*.

For some behaviors, there is considerable justification for studying the behavior in animals for its own sake, while for other behaviors, the primary, and perhaps only, justification for studying the behavior is to better understand human behavior. It should be obvious, at this point, that many, many examples exist of behaviors in animals that are useful for modeling, predicting, or understanding human behaviors. In fact, it is probably difficult to think of a human behavior that could not be studied using an animal model with some face validity. Even many of the most sophisticated (language, learning, culture) and the most abnormal (self-aggression, child abuse) patterns of human behavior can be studied and/or induced in animals. In conclusion then, we would know considerably less about our own behavior if it were not for studies of the behavior of animals.

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CHAPTER 3

Animal Models in Biodefense Research

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INTRODUCTION

The idea of attacking enemies through the use of biologic agents is an ancient one, and includes documented attempts to contaminate the wells and reservoirs of enemies with cadavers and animal

carcasses¹ and attempts to infect Native Americans with smallpox via contaminated blankets offered as gifts.² Awareness and concern over biological weapons has increased greatly as the technological sophistication required to produce related agents has become more global. As a result, interest has greatly expanded in research to more precisely define and understand agents that can be potentially used as bioweapons and in methods to control, prevent, and treat disease which might result from such agents. For the purposes of this chapter, bioweapons are understood to represent live infectious agents or toxins derived from such agents.

AGENTS WITH POTENTIAL APPLICATION AS BIOWEAPONS

The U.S. Centers for Disease Control and Prevention (CDC) characterizes agents that might be potentially used as bioweapons into three categories:

Category A agents are those that can be easily disseminated or transmitted from person to person, result in high mortality rates and have the potential for major public health impact, might cause public panic and social disruption, and require special action for public health preparedness.

Category B agents are of somewhat lower priority than Category A agents and include those that are moderately easy to disseminate, result in moderate morbidity rates and low mortality rates, and require specific enhancements of CDC's diagnostic capacity and enhanced disease surveillance.

Category C agents are third highest priority and include emerging pathogens that could be engineered for mass dissemination in the future because of availability, ease of production and dissemination, and potential for high morbidity and mortality rates and major health impact.

Table 3.1 summarizes into risk categories agents commonly viewed as having bioweapon application.

Table 3.1 Categorization of Some Biologic Diseases/Agents into Risk Categories

Agent	CDC Category
Anthrax/ <i>Bacillus anthracis</i>	A
Botulism/ <i>Clostridium botulinum</i> toxin	A
Plague/ <i>Yersinia pestis</i>	A
Smallpox/ <i>variola major</i>	A
Tularemia/ <i>Francisella tularensis</i>	A
Viral hemorrhagic fevers (filoviruses, including Ebola and Marburg, and arenaviruses, including Lassa and Machupo)	A
Brucellosis/ <i>Brucella</i> species	B
Epsilon toxin of <i>Clostridium perfringens</i>	B
<i>Escherichia coli</i> 0157:H7, <i>Salmonella</i> , <i>Shigella dysenteriae</i> , <i>Vibrio cholerae</i>	B
Glanders/ <i>Burkholderia mallei</i>	B
Melioidosis/ <i>Burkholderia pseudomallei</i>	B
Psittacosis/ <i>Chlamydia psittaci</i>	B
Q fever/ <i>Coxiella burnetii</i>	B
Ricin toxin	B
Staphylococcal enterotoxin	B
Typhus fever/ <i>Rickettsia prowazekii</i>	B
Hantavirus	C
Nipah virus	C

Some of the above pathogens have comparatively wide geographical distribution, but official statistical data on diseases due to these agents are incomplete and monitoring of natural foci of these infections merits greater efforts.

ANIMAL MODELS COMMONLY USED IN BIODEFENSE RESEARCH

Animal models have proved useful for research into the biology and biodefense aspects of many of the infectious agents listed in Table 3.1. In some cases, such as with smallpox, animal models that can be infected with the specific agent do not exist, and modeling of the disease relies instead upon study using related agents to which animals are susceptible.

There is inherent risk in working with agents that can be applied as bioweapons. For this reason, caution and prudence are important; guidelines such as those formulated by the CDC should be followed when working with such agents either *in vitro* or *in vivo*.³

Desirable characteristics for animal models used in biodefense research include that they should be readily available and relatively inexpensive; genetically defined; susceptible to the human pathogen, preferably via a route of exposure that is likely to simulate the method of dissemination during an attack with biological agents; and capable of developing disease that faithfully replicates the human disease. The models described here are those for some examples of agents that could potentially be used as bioweapons.

Anthrax

Infection with the bacterial agent *Bacillus anthracis* can result in either cutaneous or systemic forms of the disease. Of the two, the systemic form is far more clinically serious. Anthrax is a zoonotic disease with worldwide distribution, though it has declined somewhat in importance as a disease of animals due to a successful livestock vaccine.⁴ Due to the stability of the agent within the environment, infection can rapidly spread across a population.

Most work involving animal models of anthrax has related to development of a more effective human vaccine against the disease. In this regard, rodents have been of primary importance. Guinea pigs, mice, and Syrian hamsters have all been described as useful animal models of infection with *B. anthracis*.^{5–8} Animals are generally challenged intramuscularly (IM), intraperitoneally (IP), subcutaneously, or by inhaled aerosol with virulent organisms. Studies are typically conducted as LD₅₀ (the dosage that is lethal to 50% of the test subjects) paradigms to assess for efficacy of vaccine or therapeutic strategies. In studies examining *B. anthracis* lethal toxin, the major virulence factor involved in anthrax, BALB/C mice were found to be more sensitive to the lethal effects of the toxin than C57BL/6J mice were, thus demonstrating the role of genetic factors in the response of animal models to challenge.⁹ A rabbit model of inhalational anthrax for vaccine development has also been described.¹⁰

Botulism

Exposure to the toxin of *Clostridium botulinum* results in severe disease characterized by paralysis of the respiratory muscles leading to dyspnea and ventilatory failure, often resulting in death. The neurotoxin at fault is one of the most toxic substances known to produce severe neuromuscular paralysis, with a lethal dose being on the order of nanograms per kilogram of body weight.¹¹ Though the basic action of the toxin on neurotransmission has been studied in rats,^{12–15} mice,^{16–18} chickens,^{19,20} frogs,^{21–23} goldfish,²⁴ squid,^{25,26} and crayfish,^{27,28} research into the biomedical aspects of the toxin has utilized fewer species of animals. Most of this work has involved vaccine development in rodent models. Since, by definition, 1 international unit (IU) of antitoxin neutralizes 10⁴ mouse IP LD₅₀ of toxin, challenge studies typically involve mice exposed to IP doses of the

toxin.²⁹ Using such a model, a number of vaccines based on inactivated toxin or recombinant toxin molecules have been evaluated.^{30–32}

Plague

In the Middle Ages, epidemics of plague resulted in devastation, and the thought of the disease still evokes significant public fear and concern. Conceivably, public panic amid an outbreak could result in migration of people from affected areas, leading to further dissemination of the agent. The disease, which results from infection with *Yersinia pestis*, is worldwide and has been classified as a reemerging disease by the World Health Organization.³³ The natural history of the disease is that of a zoonotic infection resulting from contact with an infected rodent or through a bite from an infected flea vector. This form (bubonic form) of the disease results in lymphadenopathy and septicemia. The other main form of the disease is by inhalation of *Y. pestis* with resulting severe respiratory disease. The pulmonary form of plague may result from direct person-to-person transmission, in contrast to other agents such as botulinum toxin or *B. anthracis*. This latter form of the disease is of particular concern with respect to use of *Y. pestis* as a bioweapon. However, either form of the disease results in high mortality.

Most work using animal models of *Y. pestis* infection is focused on vaccine development. The standard model to investigate the efficacy of vaccines against plague is protection of the mouse in the face of subcutaneous or inhalational exposure.³⁴ Indeed, the historical “gold standard” for identifying *Y. pestis*-infected fleas has been inoculation of mice with pooled flea material with death of the mouse used as an indicator of flea infection; this method, however, has been shown to be less accurate than a method based on polymerase chain reaction.³⁵ Typically, the protective ability of vaccines or other therapeutic strategies is measured using an LD₅₀ design. Mice of various genetic backgrounds, including BALB/c, C57BL/6, CBA, and CB6F1, have been successfully used in studies involving systemic or inhalational challenge.^{34,36,37} In related experiments, severe combined immunodeficient (SCID)/beige mice reconstituted with lymphocytes from immunized BALB/c mice have been used to demonstrate passive protective immunity against inhaled and systemic infection with *Y. pestis*.³⁸

Other species have been used to a limited extent in studies examining *Y. pestis*. Nonhuman primates have been used to investigate the infectivity and immune response to *Y. pestis*.^{39,40} For example, African green monkeys were used in survival studies following inhalational exposure to *Y. pestis*.⁴⁰ Those studies used nonhuman primates to evaluate a pigmentation-deficient *Y. pestis* strain similar to one that stimulated protective immunity in humans vaccinated by aerosol, but not in mice. Gerbils and guinea pigs were infected IP with *Y. pestis* extracts to study purine metabolism of animals challenged with *Y. pestis* components.⁴¹

Smallpox

Variola is a highly infectious virus, thus giving it great importance as a potential bioweapon agent. Attempts to establish an animal model that faithfully replicates human smallpox have been largely unsuccessful; there are no known animals that are naturally infected with the variola virus, the causative agent of smallpox. The greatest success has been a model that involves infecting cynomolgous macaques with very high doses of the virus through a combination of intravenous injection and inhalational exposure. In that model, fatal disease following a clinical course similar to smallpox in humans is produced.^{42,43} Intracerebral inoculation of 2- to 6-day-old mice established infection without clinical signs and has been used as a system to evaluate the efficacy of specific antiviral compounds.^{44,45}

Vaccinia virus infections are probably the most common models for variola infection and have been performed not only in mice, but also in rabbits and monkeys. Many different types of vaccinia

strains have been used in animals; some of these result in only mild infections, and others cause lethal disease. As described below, models that produce skin lesions or keratitis are nonlethal; other models are frequently lethal to the animal.

The vaccinia virus mouse model takes several forms. One model involves production of tail lesions in mice following either intravenous (IV) inoculation of vaccinia virus^{46–48} or pricking of the tail with an infected needle.⁴⁹ In the former, a lesion develops at and spreads from the wound site; in the latter, dermal lesions develop along the length of the tail. Another model involves vaccinia infection in mice by intracerebral inoculation.^{50–52} Intravenous administration of vaccinia virus to SCID mice resulted in dermal tail lesions and death of the animals.⁵³ A more relevant model is the intranasal (IN) exposure of mice to vaccinia virus; that approach results in disease and death, similar to human smallpox.^{54,55}

Vaccinia virus skin lesions can be induced in rabbits⁵⁶ and nonhuman primates.⁵⁷ Similarly, keratitis models of vaccinia infection in rabbits^{58,59} and nonhuman primates⁶⁰ have found utility as models of variola infection. Both the skin models and the keratitis models have been used to evaluate efficacy of compounds developed as antivariola agents.

Other orthopoxviruses have been used in animals as models of variola infection. In this regard, cowpox, vaccinia, and rabbitpox have all been used in mice.⁶¹ Cowpox virus is lethal when administered to mice either IN or by inhalation.^{62,63} Nonhuman primates infected with monkeypox by aerosol at a sufficiently high dose die from infection, and rabbits inoculated IN with rabbitpox develop a generalized, fatal infection.⁶⁴ Though ectromelia could also be used as a model for variola infection, there is great reluctance to do so, given the high potential for spread throughout an animal facility.^{65,66} While all these models involving vaccinia virus, cowpox, monkeypox, rabbitpox, and ectromelia reasonably imitate smallpox in humans, they all fall short in that they do not derive from infection with variola virus.

Tularemia

Tularemia is a serious zoonotic disease resulting from infection with the bacterium *Francisella tularensis*. The ease with which the infectious agent can be transmitted by aerosol accounts for concern over its use as a biological weapon. Common natural reservoirs of the agent include wild rodents and lagomorphs, though it is possible to infect a much wider range of species. For example, infection with *F. tularensis* has been described in voles,⁶⁷ squirrel monkeys,⁶⁸ a common marmoset,⁶⁹ and prairie dogs.^{70,71} Epizootics characterized by high mortality have also been noted in populations of small wild mammals.

Most research requiring the use of animal models of *F. tularensis* has utilized mice infected by various routes, including IV, IP, SC, inhalation, or dermal abrasion.^{72–75} Studies directed toward development of vaccine or therapeutic strategies often use death as an endpoint or tissue bacterial load as indicators of efficacy. With low-dose *F. tularensis* challenge, it appears that systemic rather than pulmonary infection is the likely cause of death.⁷⁵ Rats^{76,77} and guinea pigs⁷⁸ have been used to a limited extent as animal models of *F. tularensis* infection.

Viral Hemorrhagic Fevers

Several important viruses result in severe disease in humans, characterized by acute febrile illness with hemorrhagic manifestations and often culminating in death. Viruses of interest include the filoviruses Ebola and Marburg and the arenaviruses Lassa and Machupo. They are all readily spread by aerosol.

Nonhuman primates are readily infected both in the wild and in captivity with Ebola and Marburg viruses;^{79–85} however, they are not believed to be the primary natural reservoir.⁸⁶ Nonetheless, nonhuman primates have been used as animal models for study of Ebola virus pathogenesis

and to develop vaccination strategies. Cynomolgous monkeys infected subcutaneously developed disease which closely resembled the human disease.⁸⁷ Similarly, Marburg virus is highly pathogenic and lethal when administered to African green monkeys.^{88,89}

Mice and guinea pigs have also been used extensively for research involving the filoviral agents of hemorrhagic fever. Mice can be readily infected by IP or SC administration of Ebola virus with resulting severe disease and death.^{90–93} A mouse-adapted strain of Ebola virus caused death when as few as 1 virion was administered IP, but mice were resistant when administered much larger doses by the SC, IM, or intradermal (ID) routes.⁹⁰ Guinea pigs are also a standard model for studies requiring an animal model of filovirus infection. Indeed, Marburg virus was first isolated by inoculation of blood and tissue samples from human patients into guinea pigs.^{86,94} Guinea pigs have been used for Ebola virus vaccine development^{95,96} and for study of the pathogenesis of both Ebola virus⁹⁷ and Marburg virus.⁹⁸ As in mice, both viruses are highly pathogenic in guinea pigs.

Guinea pigs are the primary animal model for arenavirus infections. Pichinde virus infection of guinea pigs is frequently used as a model for human Lassa fever.^{99–101} A high-passage virulent variant of Pichinde virus produces a disease in guinea pigs that mimics human Lassa fever in many ways, particularly the correlation between viremia and clinical outcome^{102,103} and a terminal vascular leak syndrome.¹⁰⁴

Other species have also been used to model arenavirus infection and disease. Machupo virus infections of mice have been used to evaluate immunologic parameters of the disease.¹⁰⁵ Mice¹⁰⁶ and nonhuman primates¹⁰⁷ have been used to evaluate treatment and vaccine strategies when challenged with Lassa fever virus. Other model systems for arenavirus infections include intracranial challenge of mice with lymphocytic choriomeningitis virus as a model system for Lassa fever virus;¹⁰⁸ SC inoculation of guinea pigs with Venezuelan hemorrhagic fever virus as a model for arenaviral hemorrhagic disease;¹⁰⁹ IV inoculation of rhesus macaques with lymphocytic choriomeningitis virus as a model for human viral hemorrhagic disease;¹¹⁰ and infection of Syrian golden hamsters with Pirital virus as a model for study of the pathogenesis of arenavirus infections.¹¹¹

Brucellosis

Brucella species are facultative intracellular pathogens that localize to cells of the reticuloendothelial system and whose host range includes livestock, dogs, and humans. *Brucella* is very stable in the natural environment. The human disease, sometimes referred to as Malta fever, is a very serious public health problem and is characterized clinically by fever, malaise, headache, and arthralgia. The infection can be slowly progressive and is associated with prolonged disease and disability.

Experimentally, a number of studies have been done using the natural hosts of the infection, including goats^{112–114} and cattle;¹¹⁴ however, although these models are useful they are typically used to specifically study the disease with an interest in the specific application of the data to the veterinary needs of those species.

Rodents are the most commonly used animal model of brucellosis. Studies typically are designed to evaluate the splenic weight and/or bacterial load following immunization^{115–117} or treatment with a test compound,¹¹⁸ though other studies have used the model for research into the basic pathophysiology of *Brucella*.^{119–121} For vaccination studies, immunologic parameters are also frequently measured. Infection of mice with *B. abortus* administered IV has been used as a model for chronic fatigue syndrome.¹²² A rat model of brucellosis induced by IP administration of *B. melitensis* has also been described¹²³ and involves measurement of hepatic and splenic weights and bacterial loads following infection and experimental treatment.

Enterotoxemia Due to Epsilon Toxin from *Clostridium perfringens*

Clostridium perfringens strains B and D elaborate a 30,000-MW protein toxin that is an agent of food poisoning in humans and enterotoxemia in livestock, particularly lambs, though it can also be readily dispersed by aerosol. The toxin is quite powerful, with an IV LD₅₀ of 0.1 µg/kg in mice.

The mouse lethality assay is a common animal research model for studies into the pathogenesis and treatment of *C. perfringens* epsilon toxin enterotoxemia.^{124,125} Studies are run as survival studies with the goal of establishing the LD₅₀ in response to experimental intervention following IP administration of the toxin. This assay is also sometimes referred to as the mouse neutralization test when the potency of vaccines is the subject of investigation.^{126,127} Enzyme-Linked Immunosorbent Assay (ELISA) methods to replace the mouse neutralization assay are finding application in some laboratories.¹²⁸

Goats and sheep have also been used to a great extent in studies of epsilon toxin, particularly studies into the pathogenesis of the resulting disease.^{129–132} Typically, sublethal doses of the toxin are administered IV. Lambs and kids tolerate greater dosages than mice do¹³⁰ but will develop severe, generalized vasogenic edema leading to death with larger doses.¹³¹ The effects of epsilon toxin have also been studied in ligated intestinal loops of goats and sheep, where it was suggested that the toxin modifies ion and water transport through different mechanisms.¹³²

Other species of animals have been sporadically used as models for studies involving epsilon toxin. Guinea pigs have been used as a model for studying the clinical and pathological changes associated with the toxin.¹³³ Both IP and intradermal inoculation of crude toxin preparations were used to characterize the model. Following IV inoculation of toxin, calves have been shown to show some, but not all, of the histological features demonstrated by sheep and goats.¹³⁴

Typhoid Fever and Enteritis Associated with *Salmonella enterica*

In humans, *Salmonella enterica* serotypes have been associated with two common disease syndromes, enteritis and typhoid fever. Typhoid fever results from infection with serotypes that are specifically adapted to humans or higher primates, while enteritis may result from infection with any of more than 2500 serotypes. Disease generally results from peroral exposure, thus the use of *S. enterica* as a bioweapon would likely arise via contamination of food or water supplies.

The mouse is the primary model of typhoid fever.¹³⁵ Serotype Typhimurium is a natural pathogen for rodents, with infection resulting in typhoid-like disease in mice and a tissue distribution of bacteria similar to that in humans. The mouse model has proved quite useful for elucidating virulence mechanisms of and host resistance to this pathogen.^{136–139} Balb/c is a commonly used line for studies requiring an animal model of typhoid fever. The C57Bl6 is also susceptible to infection, but the CBA has been shown to be resistant.¹⁴⁰ Typically, the mouse is challenged perorally with an infectious dose of the organism. Within 4 to 8 days following oral administration of live bacteria, mice develop clinical disease characterized by pyrexia, lethargy, and an unkempt appearance; however, in contrast to the human disease, mice do not develop diarrhea. Infection in mice is widely systemic, whereas in humans the infection is usually more localized to the intestinal tract. Typhoid fever closely resembling the human disease can be produced in chimpanzees by oral infection with serotype Typhimurium;¹⁴¹ however, cost and logistical issues with handling great apes limit use of this model.

Calves are a widely used model of *Salmonella* enteritis. Both Dublin and Typhimurium serotypes result in enteritis in calves; however, the disease resulting from peroral infection with Typhimurium is the better model of the human disease since Dublin is more invasive, and infection may manifest as meningoencephalitis, polyarthritis, osteomyelitis, and pneumonia.¹⁴² In contrast, oral infection of calves with Typhimurium results in diarrhea, pyrexia, and dehydration. Both calf¹⁴³ and rabbit¹⁴⁴ ligated ileal loop models have also been used to study the virulence factors associated with *Salmonella* enteritis; these models are of use primarily for studying early phases of the infection. Rhesus macaques¹⁴⁵ and rats^{146–148} have also been used to a limited extent for *Salmonella*-associated enteritis. In contrast to the mouse, the small intestine is the main site of *Salmonella* serotype Typhimurium colonization in the rat. However, the rat does not readily develop clinical disease following peroral infection and thus is of primary use for studies investigating strategies designed to block colonization of the intestinal tract. The chicken has also been used as a model for investigations into the virulence of *Salmonella*.¹³⁹

Shigellosis

Shigella spp., primarily *S. dysenteriae*, are associated with acute bacterial enteritis in humans with two intestinal presentations, diarrhea and dysentery. *Shigella dysenteriae* produces a toxin (Shiga toxin) that has been implicated in the pathogenesis of the disease.

Humans and nonhuman primates are the only natural hosts for shigellae. However, because of the expense and logistical difficulties associated with research involving nonhuman primates, the more common animal model is the guinea pig. In particular, the guinea pig Sereny test is used to assess the invasive ability of shigellae^{149,150} and to assess protective immunity following vaccination.¹⁵¹ The Sereny test involves inoculation of the conjunctival sac of a guinea pig with pathogenic shigellae; the degree of invasiveness is then evaluated by the degree of keratoconjunctivitis associated with invasion of corneal epithelial cells and infiltration with polymorphonuclear cells, similar to that seen in human intestinal lesions caused by shigellae. Mice have also been used as models in the Sereny test.¹⁵² The Sereny test is often regarded as a system that likely results in significant pain and distress to the test animal; for this reason, buprenorphine has been evaluated as a measure to provide analgesia for such animals.¹⁵³ Although treatment with buprenorphine did not influence the outcome of immunologic evaluations, significant changes in volume of ocular discharge and body weight were noted.

Rabbits infected perorally with *Shigella flexneri* have been used as models for human shigellosis.^{154–156} One such model system involves a preinoculation conditioning procedure that includes a 36-h fast as well as doses of oral tetracycline, intravenous cimetidine, and intraperitoneal tincture of opium.¹⁵⁷ A rabbit model produced by direct colonic infection with *S. flexneri* combined with cecal ligation has also been described.¹⁵⁸ Studies into the pathophysiology of and immune response to Shiga toxin–associated disease have been conducted in the rabbit ileal loop model.^{159–161}

SCID mice with human intestine xenografts have been used as models for infection with *Shigella*.¹⁶² This approach allows direct examination of the interaction between shigellae and human intestine, albeit in an artificial system.

Escherichia coli 0157:H7 Syndromes

Enterohemorrhagic *Escherichia coli* (EHEC) is associated with severe hemorrhagic colitis and hemolytic uremic syndrome. Adherence of EHEC to the intestinal epithelium produces characteristic attaching/effacing lesions in the intestines of gnotobiotic piglets, infant rabbits, and chickens,^{163–165} but not in mice or humans.^{166–169} Toxins similar to shiga toxin (shiga-like toxins 1 and 2) are considered to be important virulence factors of EHEC.¹⁷⁰ The pathogen is naturally acquired by oral exposure, thus use as a bioweapon would likely result from contamination of water or food supplies.

A number of animal models lacking normal mammalian adult gut flora have been used to model EHEC infections, including streptomycin-treated mice,¹⁶⁵ chickens,¹⁶³ infant rabbits,^{171–174} weanling piglets,¹⁷⁵ gnotobiotic piglets,¹⁷⁶ and calves.^{177,178} Although useful in many aspects, these models do not develop hemorrhagic colitis or glomerular pathology.

Adult mice infected orogastrically develop acute renal tubule necrosis but not colitis,^{170,179} though infection of C3H mice with EHEC strains specifically producing shiga-like toxin 2 caused necrotizing colitis and glomerular lesions.¹⁸⁰ Mice, including Balb/c, C57Bl/6, and ICR lines, have been used in studies examining the immune response to EHEC.^{181,182} In contrast, colonization of the intestinal tract of CD-1 mice is only transient, and most isolates do not produce pathologic lesions or clinical disease.¹⁸³ Germ-free mice develop nonhemorrhagic colitis and glomerulitis.¹⁸⁴

Bonnet macaques (*Macaca radiata*) infected with EHEC intragastrically are a useful model of the disease.¹⁸⁵ The animals develop attaching/effacing lesions in the colon and severe epithelial damage suggestive of incipient ulcers. Moderate renal tubule changes occur, but glomerular changes are not a feature of the disease in this model.

A shiga toxin 1-producing EHEC isolate from Dutch belted rabbits was associated with a natural outbreak of bloody diarrhea and sudden death.¹⁸⁶ The disease was characterized by erosive and necrotizing enterocolitis and proliferative glomerulonephritis; thus, Dutch belted rabbits may represent a valuable animal model of the disease.

Ferrets treated with streptomycin prior to oral inoculation with EHEC have been characterized as an animal model for hemolytic uremic syndrome.¹⁸⁷ Though the animals do not develop colitis, hematuria with concomitant glomerular pathology is characteristic.

Cholera

Early references to cholera can be found in Indian writings from the ninth to seventh centuries B.C. Endemic foci in deltas of the Gang and Brahmaputra Rivers are the earliest described foci of infection, and this region of India is sometimes referred to as the “cholera motherland.”

Cholera is an acute disease characterized by severe diarrhea leading to severe electrolyte depletion, dehydration, acidosis, shock, and possibly death. The disease results from elaboration of an oligomeric protein toxin, cholera toxin, by the bacterium *Vibrio cholerae*.

The suckling mouse model is a commonly used system for the study of *V. cholerae* colonization of the intestinal tract.^{188,189} Importantly, it has been shown that *V. cholerae* expressed virulence factors in this model that are known to be important for the human disease.¹⁸⁹ A major disadvantage is that this model cannot be used for vaccine studies because the suckling mice do not survive long enough to acquire protective immunity. The most common application of the model is the competition assay, in which the suckling mouse is infected with both a mutant *V. cholerae* strain and an isogenic wild-type strain to gauge the relative importance of specific virulence factors as expressed by the mutant strain. Another common application is the LD₅₀, where lethality is measured at specific time points after inoculation; however, the short life span of infected suckling mice only allows for measurement of mortality at relatively early time points, usually up to 48 h. Infant rabbits have also been used in studies similar to those using the suckling mouse.^{190,191}

Studies examining the immune response to *V. cholerae* and to cholera toxin have been conducted in rats¹⁹² and rabbits.¹⁹³ In both the rat and rabbit models, it was demonstrated that various cholera vaccine formulations conferred protection against intestinal colonization with *V. cholerae* following orogastric inoculation of live bacteria. With high doses of bacteria, rabbits may develop fatal diarrheal disease.¹⁹³

Ligated intestinal models have been used to study *V. cholerae* in rabbits. In one such model, segments of the small intestine are ligated and suspensions of *V. cholerae* inoculated into each segment.^{194–196} Fluid accumulation is then assessed 16 to 18 h later as a measure of virulence or toxinogenicity of the bacterial strain. In the removable intestinal tie–adult rabbit diarrhea (RITARD) model,^{197–200} the intestine is ligated at the ileocecal junction and a *V. cholerae* suspension inoculated into the small intestine. Several hours later, the ligature is removed to restore intestinal patency, and the degree of diarrhea and, in some cases, number of *V. cholerae* organisms in the feces assessed over the following few days. This model is commonly used to study the intestinal pathology, intestinal bacterial colonization, and disease onset and severity of *V. cholerae* strains.

Several other approaches have been used as models to study the pathogenesis of *V. cholerae*. Germfree mice orally inoculated with *V. cholerae* are readily colonized and develop systemic and mucosal immune responses to the pathogen.²⁰¹ A murine pulmonary model in which adult mice are infected by intranasal instillation of the pathogen has been used to assess inflammation associated with *V. cholerae* infection.²⁰² Isolated porcine jejunum has been used as a model to investigate neurogenic signal transduction of cholera toxin–induced fluid accumulation.²⁰³ Larvae of the silkworm, *Bombyx mori*, have been used as a model system for evaluating the efficacy of antibiotics against several human bacterial pathogens including *V. cholerae*.²⁰⁴

Glanders

Burkholderia mallei is the bacterial agent of glanders, a zoonotic disease that does not persist in nature outside of its host, the horse.²⁰⁵ The disease is characterized by inflammation of mucous membranes and deep cutaneous ulceration that may extend into bone and cartilage. Although natural human cases of glanders are exceedingly rare, *B. mallei* is considered a potential biological weapon due to its virulence and ability to be transmitted via inhalation.

The Syrian hamster is a common animal model for the study of *B. mallei* virulence and pathophysiology of glanders.^{206–208} Hamsters are exposed to the bacteria typically by intraperitoneal injection, and disease similar to that in humans develops. Mice have also been used in similar studies, with sublethal and lethal models shown to be pathogen dose dependent.²⁰⁹

The invertebrate *Caenorhabditis elegans* has also been used as a model to study the pathogenesis of glanders.^{210,211} That model has also been used to investigate the pathophysiology of *Burkholderia pseudomallei*.

Melioidosis

A close relative of the agent of glanders, *B. pseudomallei* causes a disease (melioidosis) with clinical manifestations including asymptomatic pneumonitis, acute or chronic pneumonia, and overwhelming septicemia. Exposure to the agent is typically by inhalation.

Mice are the most common animal model used to investigate the pathogenesis of melioidosis. Balb/c mice are susceptible and used as a model of acute infection,^{212, 213} while C57Bl/6 mice are relatively resistant to infection and may therefore be an appropriate model for chronic forms of the infection.²¹⁴ Mice are typically exposed to the pathogen by intravenous or intraperitoneal injection, though whole-body aerosol exposure has been utilized in an effort to create a model that might more closely replicate human exposure in the face of an attack with a bioweapon.²¹⁵ Disease in Balb/c mice involves rapid bacteremia with death coming approximately 96 h after infection.

Psittacosis

Psittacosis is a bacterial infection of humans that can result in severe pneumonia and death. Because inhalation of aerosols is a primary mode of transmission, *Chlamydia psittaci* is regarded as having potential use as a bioweapon. Though a number of animal species including ruminants, horses, cats, amphibians, and birds harbor the causative agent, *C. psittaci*, studies focused on understanding human respiratory psittacosis have focused on the mouse as a model.

The mouse is the primary animal model for studies examining the pathogenesis of *C. psittaci*. Mice are usually exposed to the agent by intramuscular,²¹⁶ intravenous,^{217,218} or intraperitoneal²¹⁹ injection. Direct exposure to the respiratory tract has also been accomplished via intranasal inoculation for a related pathogen, *Chlamydia pneumoniae*.²²⁰ Investigations often are designed as survival studies to measure the role of virulence factors or efficacy of interventions. Intracerebral inoculation of mice with the A22 *C. psittaci* isolate from sheep has been used as a method to produce a nonlethal infection; this approach has been used as a model in studies related to vaccine development.²²¹ Various strains of mice have been used as models, with C57Bl/6 mice showing greater resistance to infection than CBA mice do.²²² Immunodeficient nude mice have been used to demonstrate the importance of γ -interferon in the pathogenesis of the infection.²²³

Guinea pigs are naturally susceptible to conjunctival infection with *C. psittaci*, and many develop conjunctivitis.²²⁴ Many investigations have used guinea pigs exposed intravaginally to the pathogen.^{225–228} Such studies are performed to evaluate the pathogenesis of *C. psittaci* reproductive tract infection rather than the respiratory tract infection that one would expect if the agent were used as a bioweapon.

Birds have been used to study respiratory infection with *C. psittaci*, albeit from the perspective of understanding avian psittacosis. In this regard, white leghorn chickens inoculated by the intra-air sac route with either avian or mammalian strains of the pathogen developed generalized infection, though mammalian strains were far less pathogenic.²²⁹ One-day-old Japanese quail inoculated by the intra-air sac route with an avian strain of *C. psittaci* developed lethal infection; in contrast, 7-day-old birds were more resistant to infection and disease.²³⁰ Chicken embryos have been used as models to examine the efficacy of antibiotics against *C. psittaci*.²³¹

Q Fever

Q fever is caused by an obligately intracellular bacterium, *Coxiella burnetii*. It is found worldwide except in New Zealand.²³² The human disease has both acute (mainly as a febrile illness or pneumonia) and chronic (mainly endocarditis) forms and is typically acquired from domestic or wild animals; many cases originating from contact with infected sheep have been documented. It is difficult to diagnose Q fever based upon clinical symptoms alone; thus, diagnosis is usually based on serologic testing. Because humans typically acquire the infection by inhalation of the agent within aerosols, there is potential for *C. burnetii* to be used as a bioweapon.

Many species of mammals and birds are reservoirs of *C. burnetii* in nature. Sheep are often implicated in the transmission of the disease to humans.^{233–235} In central Russia, a large epidemic of Q fever in humans was associated with goats as the source of infection.²³⁶ Most often, *C. burnetii* infection is latent in animals, with persistent shedding into the environment. With chronic infections, abortions have been noted in sheep and goats^{237,238} and lower birth weights and infertility in cattle.^{239,240} Humans are usually infected via contaminated aerosols from domestic animals. Person-to-person transmission is extremely rare. *Coxiella burnetii* is very resistant to physical factors and is stable in the environment; it may therefore survive for several weeks in areas where infected animals were present. The agent can be disseminated by air currents;^{241,242} infection may therefore occur in patients having no contact with animals.

The guinea pig is susceptible to infection and disease following exposure to *C. burnetii* by either inhalational^{243,244} or intraperitoneal²⁴⁵ routes. Acute Q fever in humans very closely resembles that modeled by the guinea pig model.²⁴⁶ Investigations are typically run as survival studies to investigate the pathophysiology of infection or the efficacy of intervention. In guinea pigs, systemic manifestations of the disease, such as fever, weight loss, hypoglycemia, and other metabolic disorders, have a distinctive cyclic nature coincident with the cyclic development of infectious foci. In these animals, *C. burnetii* infection is characterized by fever that appears 3 to 10 days after infection and continues for up to 2 weeks, depending on the *C. burnetii* dose and route of transmission. During the febrile state, *C. burnetii* can be isolated from the blood of guinea pigs, with the chances of isolation greater during the first days of the fever.

Guinea pigs may recover from the disease within 2 to 3 weeks when induced with small doses of *C. burnetii*. In contrast, high doses of the bacterium (i.e., greater than 10^9 *C. burnetii* cells) result in acute death of all infected guinea pigs. Pathological changes in various tissues are present within days of infection and can persist for several weeks.^{247,248} Typical lesions include splenomegaly, lymphadenopathy, mononuclear inflammation in the lungs, and granulomatous inflammation of the liver, spleen, and bone marrow.

Infected animals usually develop acute pulmonary or hepatic disease. Guinea pigs have also been used to examine delayed-type hypersensitivity reactions associated with Q fever vaccines;²⁴⁹ in such studies, vaccines are administered either subcutaneously or intradermally to hairless guinea pigs and the subsequent presence or absence of dermal granulomatous reactivity noted. To study Q fever endocarditis, a frequent outcome of chronic *C. burnetii* infection, a guinea pig model involving intraperitoneal inoculation with the pathogen following electrocoagulation of the aortic valves was used.²⁵⁰

As for many pathogens, mice have been used extensively to model infection with *C. burnetti*. Inoculation by either the intraperitoneal or intranasal routes results in infection and pneumonia.²⁵¹ Following IP inoculation of mice, the organism spreads rapidly to various tissues resulting in microabscesses and granulomas in the spleen, liver, kidneys, and adrenal glands.^{252–254} Splenomegaly is characteristic, with the weight of the spleen directly correlating with *C. burnetti* dose.²⁵⁵ The lesions may persist for several months, with the mice remaining chronically infected. *Coxiella burnetti* DNA was detected in the hearts, spleens, and kidneys of mice within 6 hours of intraperitoneal administration and persisted in these tissues for up to 214 days.²⁵⁵ Reactivation of infection may result following treatment with steroids²⁵⁶ or cyclophosphane.^{257,258} Infection may be mild or severe, even resulting in death depending upon the dose and strain of *C. burnetti*.²⁵⁹ A/J mice are considered relatively more susceptible to infection than the more resistant C57Bl/6J strain.²⁶⁰ Balb/c mice are a useful model of sublethal infection for evaluation of vaccine protective efficacy.²⁵⁴ Immunodeficient nude mice exposed to *C. burnetti* by aerosol developed similar degrees of infection as euthymic mice did,²⁶¹ though nude mice clear the organism from peripheral circulation and the spleen more slowly.²⁶² Some strains of *C. burnetti* are capable of long-term persistence, with one study demonstrating persistence through three generations of mice.²⁶⁴

Some investigations have used nonhuman primates for studies into Q fever pathogenesis and vaccination strategies. Both rhesus monkeys and cynomolgous monkeys are susceptible to infection and disease, with radiologic evidence of pneumonia following aerosol exposure to *C. burnetti*.^{265,266} Cynomolgous monkeys challenged by aerosol with *C. burnetti* were used to demonstrate efficacy of a noncellular vaccine.^{266,267}

Ricin Toxicosis

Ricin is a potent toxin, derived from the castor oil plant (*Ricinus communis*), which inhibits protein synthesis. When inhaled, ricin produces severe damage to the respiratory tract without apparent systemic toxicity. Microscopic features of the damaged lung include perivascular, interstitial, and alveolar edema and inflammation.

Mice used in aerosol challenge studies are the common animal model to study the pathophysiology of ricin and possible preventive strategies. This method allows direct exposure of peripheral alveoli in contrast to the intratracheal or intranasal instillation methods, in which the toxin fails to reach peripheral lung fields.²⁶⁸ Using this model, intramuscular,²⁶⁹ intranasal,²⁷⁰ or oral²⁷¹ immunization with ricin toxoid and treatment with aerosolized antiricin antibody²⁷² have been shown to be effective preventive strategies. NIH-Swiss mice^{269–272} and CD-1 mice^{271,272} are commonly used in such studies.

Rats have been used similarly to mice, with exposure to ricin-containing aerosols used as a means of exposure. The Porton strain of Wistar rat is commonly used in such studies.^{273–276} Ricin toxoid delivered subcutaneously²⁷³ or intratracheally^{274–276} stimulates various degrees of immunity against aerosol challenge with ricin.²⁷⁴ Intraperitoneal administration of ricin to Wistar rats has been used as a model to show the potential for ricin to induce hepatic damage following exposure by a noninhalation route.²⁷⁷

Staphylococcal Enterotoxigenesis

Staphylococcal enterotoxins cause a generally self-limiting gastrointestinal intoxication and are a common cause of food poisoning. In contrast, parenteral exposure, particularly through inhalation of aerosols, can cause a potentially fatal shock syndrome.

Nonhuman primates have been the accepted animal model for the study of staphylococcal enterotoxin B–induced toxicity. Rhesus monkeys exposed to aerosols of the toxin initially experience a gastrointestinal syndrome characterized by anorexia, vomiting, and diarrhea progressing to respiratory distress, shock, and death.^{278–281} The pulmonary lesions are consistent with increased

capillary permeability and include alveolar and interstitial edema and fibrin exudation into the alveolar space. Vaccination with a toxoid confers protective humoral immunity to aerosol challenge.^{282–284}

Mice have also been used to model staphylococcal B-enterotoxigenesis following aerosol exposure. Most models using mice require pretreatment of the animals with various agents to increase susceptibility. Examples of such agents include D-galactosamine,²⁸⁵ actinomycin D,²⁸⁶ and lipopolysaccharide.^{281,287,288} Following exposure to staphylococcal enterotoxin-containing aerosols, histologic lesions in the lungs of lipopolysaccharide (LPS)-treated mice are similar to those of nonhuman primates.²⁸¹ In contrast, intranasal exposure of C3H/HeJ mice to the toxin with no pretreatment results in disease that resembles that of nonhuman primates.²⁸⁹ Intraperitoneal administration of staphylococcal enterotoxin B has also been used as a route of exposure in mice.^{290,291}

Because the influence of staphylococcal enterotoxins on vascular permeability leading to pulmonary edema is a major factor in the pathophysiology of the disease, the edematogenic response to the toxin is of interest. In this regard, the mouse hind-paw edema test has been used to study this effect.^{292,293} Typically, a small dose of the toxin is administered to the intraplantar tissues of mice, and the paw weight and exudation of Evans blue dye into the tissue evaluated within several hours.

Other species have been used infrequently to model staphylococcal B-enterotoxigenesis, including perorally exposed ferrets²⁹⁴ and intramuscularly exposed rabbits.²⁹⁵

Typhus Fever

Rickettsia prowazekii, the causative agent of typhus fever, is spread between humans primarily via body lice. The resulting disease is characterized by hepatic and renal dysfunction, central nervous system abnormalities, and pulmonary compromise. The potential for the use of *R. prowazekii* as a bioweapon was explored by the Red Army of the former Soviet Union and the Japanese Army, which tested biobombs containing *R. prowazekii*.²⁹⁶

Although the eastern flying squirrel (*Glaucomys volans*) is a known reservoir of *R. prowazekii*, it is generally not used as an animal model for infection aside from limited studies examining the potential for infection of different arthropod vectors.²⁹⁷ A number of species can be infected with the pathogen but do not develop disease. For example, rabbits,²⁹⁸ cotton rats,^{299–301} and guinea pigs³⁰² can all be infected but with no resulting illness. Dogs inoculated with *R. prowazekii* seroconverted but did not become infected with the agent.³⁰³

Although some investigators have found that mice infected with *R. prowazekii* do not develop disease,^{304,305} others have demonstrated that mice infected with large doses of *R. prowazekii* develop severe pneumonia in 3 to 4 days with subsequent death of the animals.³⁰⁶

It appears that the best model for human typhus fever is the cynomolgous monkey. Animals infected intravenously developed clinical illness and pathological changes characteristic of typhus in humans.³⁰⁷

Hantavirus Infections

Hantaviruses have long been associated with hemorrhagic fever with renal syndrome in Korea, China, Russia, and Europe.^{308,309} In the western United States, Sin Nombre hantavirus is the etiological agent responsible for hantavirus pulmonary syndrome. Exposure is believed to typically occur by inhalation of aerosols containing infectious virus shed in the feces of endemically infected rodents.

The primary reservoir of Sin Nombre hantavirus is the deer mouse (*Peromyscus maniculatus*), and this species has been used as a model to study the infection and the efficacy of potential vaccines.^{310,311} In this model, animals are typically challenged intramuscularly with the virus. It is

important to recognize, however, that the deer mouse does not develop disease or pathologic lesions as the result of infection with hantavirus.

A variety of hantaviruses can produce infections in laboratory animals but with no resulting clinical disease or pathologic changes. For example, Puumala hantavirus is a European-originated agent that infects bank voles.^{312,313} Hantaan, Seoul, and Puumala hantaviruses all infect rats by inhalation or intramuscular inoculation.³¹⁴

Nonhuman primates have proved valuable as models of hantavirus infection and disease. *Cynomolgus* monkeys exposed to the Andes hantavirus either intravenously or by inhaled aerosol became infected and seroconverted but did not develop clinical disease.³¹⁵ Because this is the only known model of infection with a hantavirus pulmonary agent in a nonhuman primate, it has utility for investigations focused toward vaccine development. In contrast, when infected with Puumala hantavirus, *cynomolgus* monkeys developed disease characteristic of hantavirus hemorrhagic fever and renal syndrome.³¹⁶

The Syrian hamster has proven to be the best model for hantavirus pulmonary syndrome. Following infection with Andes hantavirus, hamsters quickly develop rapidly progressing respiratory distress, with pulmonary edema and pleural effusion similar to the human disease.³¹⁷ This model has been used to demonstrate the efficacy of immunoprophylaxis for infected hamsters using immune serum.³¹⁸

Hantaan virus, the etiological agent of Korean hemorrhagic fever, has been shown to cause an acute, fatal disease in mice following intraperitoneal inoculation. Mouse strains including C57Bl/6, Balb/c, AKR/J, and SJL/J all rapidly developed neurological disease characterized by paralysis and convulsions.³¹⁹

Nipah Virus Infection

Nipah virus is a newly emerged paramyxovirus that resulted in hundreds of infected patients in Malaysia.³²⁰ Clinical disease ranges from fever and headache to a severe acute febrile encephalitic syndrome. Though the fruit bat is believed to be the natural host, pig-to-human transmission through close contact was established as a principal means of transmission.³²¹

Naturally and experimentally infected pigs and cats demonstrated vasculitis but not encephalitis.³²² In contrast, following either intraperitoneal or intranasal inoculation, the Syrian hamster developed clinical disease and pathologic changes resembling those seen in humans.³²³ The lesions observed were most severe in the brain and included vasculitis and thrombosis.

SUMMARY

Though the use of biological agents as weapons is an ancient concept, the notion that preemptive strategies can be used to defend against such weapons is relatively new. Animal models represent basic tools to study and define infection and disease resulting from use of such weapons. The need for continued research is critical, and the U.S. Food and Drug Administration recently exercised its power to approve critical drugs and vaccines based on their effectiveness in animal tests rather than on extensive human studies.³²⁴ This move, meant to hasten the development and availability of drugs for protection against bioweapons, not only allows bypass of human efficacy studies (but not human safety studies) but also mandates expanded studies in animals. Actions such as this underscore the important role animals will continue to play in biodefense research.

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CHAPTER 4

Animal Models in AIDS Research

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INTRODUCTION

The acquired immunodeficiency syndrome (AIDS) is a global epidemic caused by one of two related retroviruses of the lentivirus family, human immunodeficiency virus (HIV) types 1 and 2. Most cases of AIDS worldwide are due to HIV-1 infection, but cases due to HIV-2 infection are more prevalent in certain areas of Africa. According to reports from the United Nations Program on HIV/AIDS (UNAIDS) in 2002, 42 million people are living with HIV-AIDS, and 5 million new cases are reported every year throughout the world. Most AIDS-related complications and deaths

occur in sub-Saharan Africa, but Asia now accounts for 15% of all infections, with an estimated 3.9 million cases reported in India alone. Important strides have been made in the treatment of HIV infection with highly active antiretroviral therapy (HAART). However, this therapy is expensive and therefore is accessible mainly to people in developed countries. Drug-related toxicity and the development of drug-resistant strains are the main problems associated with HAART that hinder progress in curtailing this epidemic.

The most cost-effective approach against infectious diseases in general, and HIV-AIDS in particular, is preventive or prophylactic vaccination. The best historical examples of successful vaccine-mediated control of viral infections are measles, polio, and hepatic diseases associated with hepatitis B viruses. Yet, many of the vaccine strategies successful in controlling other viral infections have been ineffective in controlling HIV-AIDS, perhaps because HIV-1 infection causes depletion of CD4⁺ T cells that are central to the adoptive immune system.¹ Also, since HIV-1 infection is chronic and lifelong, and the host's immune responses are for the most part misdirected, there is potential for immune exhaustion and failure to control or clear the virus.¹ Thus, despite widespread efforts, currently no vaccine candidates exist to effectively prevent infection or serve as therapy for HIV-induced disease. One major hurdle for progress in this regard is the lack of a perfectly suited animal model for directly testing vaccine candidates derived from HIV antigens. Nevertheless, animal models have provided useful insights into different aspects of HIV infection, pathogenesis, and host immunity.

This chapter summarizes the knowledge gained in recent years from various animal models, particularly primate models because of their genetic and physiologic closeness to humans and their general usefulness in planning human clinical trials of HIV vaccines and therapeutic strategies. First discussed, however, is the available information on infection by HIV-1 and closely related members of the lentivirus family: equine infectious anemia virus (EIAV), bovine immunodeficiency virus (BIV), caprine arthritis–encephalitis virus (CAEV), Maedi–Visna virus (MVV), feline immunodeficiency virus (FIV), simian immunodeficiency viruses (SIVs), and laboratory-constructed strains of simian–human immunodeficiency virus (SHIV). For simplicity, ease of manipulation, and economics, a small-animal model for HIV-AIDS would be ideal, and efforts by some laboratories using transgenic mice and rats and mice with severe combined immunodeficiency (SCID mice) are explained. However, an in-depth survey of studies describing disease characteristics for HIV or related viruses is beyond the scope of this chapter. Because several related topics have been covered in recent reviews,^{1–5} only new data obtained in the last few years from animal models for HIV-AIDS in humans are highlighted herein.

DESIRABLE FEATURES OF AN IDEAL ANIMAL MODEL FOR HIV-AIDS

An ideal animal model for HIV-AIDS in humans would involve HIV or a close relative such that the host range, tissue distribution, disease progression, and route of infection are similar, if not identical. However, the disease course should be relatively shorter in the animal so that efficacy testing can be completed in a reasonable time, thereby allowing rapid transition to human clinical testing. Most important, the immune correlates for protection should be easily translatable despite the differences in genetic makeup between the animal and human. Finally, the chosen animal model should be easy to work with, relatively inexpensive, and free of regulatory constraints, and the animals must be easily obtained in adequate numbers.² An animal model with all or most of these desired characteristics would quickly enable the design of vaccines and therapeutic strategies amenable to humans.

Such an ideal model has not yet been identified or created, so researchers use a variety of animal models involving infection by closely related lentiviruses to mimic various aspects of HIV-1 infection, disease progression, and the nature of protective immunity. For example, EIAV, BIV,

CAEV, and MVV are used to study viral replication and persistence in the host and FIV in cats and SIV in monkeys to understand lentivirus-induced neuropathology and immune dysregulation. More recently, researchers have employed the chimeric SHIV constructed in the laboratories by combining the *gag*, *pol*, *vif*, *vpr*, and *vpu* genes and the long terminal repeat (LTR) sequences of SIV, and the *tat*, *rev*, and *env* genes of HIV. Macaques infected with SHIV exhibit AIDS-like disease, including systemic and neurological symptoms similar to those observed in humans after HIV-1 infection. Because SHIV encodes the gene for the HIV envelope and relies on the envelope protein to infect macaques, several laboratories have used SHIV as a surrogate for testing HIV envelope-based vaccines and therapeutic strategies. This chapter summarizes the advantages and disadvantages of each of these viruses in their corresponding animal hosts and comments on the potential for translation or extrapolation of the findings to humans.

EQUINE INFECTIOUS ANEMIA VIRUS (EIAV) INFECTION IN HORSES AS A MODEL

The natural host range for EIAV infection includes horses, mules, donkeys, and other equine species. The virus is transmitted by biting insects and by contaminated instruments and needles. Persistent viremia is observed in EIAV-infected animals despite the host mounting antiviral immunity.⁶ EIAV is the only lentivirus associated with an immune complex disease. The primary target cells are macrophages and erythroid precursors, but unlike in the case of HIV-1 infection, the CD4⁺ T cells are not infected and the secondary infections common to HIV-AIDS are not observed. Even so, the immunopathogenesis of HIV and EIAV similarly affects the immune system of the host.⁷ The symptoms associated with EIAV infection range from a fatal acute syndrome with severe anemia to a chronic syndrome of relapsing anemia, wasting, renal immune complex disease, and encephalitis. Carriers may have no visible signs of disease until stress or steroids trigger relapses of symptoms.

The EIAV-infected horse is considered a relevant model of HIV-AIDS in humans because protective immunity includes cellular immune responses responsible for clearance of both EIAV-infected cells and HIV-infected cells and because mutations in the *env* gene sequences during the course of EIAV infection resemble those reported for sequential isolates of HIV in patients.⁷ However, the large size of equines limits wider utility of this model in laboratory-based studies.

BOVINE IMMUNODEFICIENCY VIRUS (BIV) INFECTION IN CATTLE AS A MODEL

BIV was originally isolated and identified in cattle, but the natural host range for the virus also includes sheep and goats. Like HIV, BIV exhibits mother-to-offspring transmission, persistent infection, and genomic variation.^{8–10} The circulating virus falls to undetectable levels after initial replication, similar to that seen during the asymptomatic phase of HIV-1 infection in humans. However, unlike with HIV, no symptoms are usually associated with BIV in naturally infected cattle or in experimentally infected sheep, and in only some cases have lymphocytosis, lymphadenopathy, wasting, and encephalitis been noticed.¹¹ Also, no immune disturbances and no secondary infections have been observed in cattle after BIV infection.

Recently, a BIV/HIV-1 chimeric virus (HBIV₃₇₅₃), which expresses the 5' and 3' LTR sequences of HIV and the *gag-pol* region of BIV, has been developed to provide an improved model for the study of AIDS in cattle.¹² However, as in the EIAV model, the size of the host animal for BIV poses obstacles for large-scale studies.

Experimentally, rabbits can be infected with BIV, and some studies have attempted to establish the BIV-infected rabbit as a useful animal model for HIV-AIDS.^{13,14} BIV-infected rabbits show impaired B lymphocyte and T lymphocyte functions that coincide with clinical disease, including immunodeficiency, anorexia, weight loss, neurological impairment, splenomegaly, and mesenteric

lymphadenopathy. BIV can also replicate at mucosal surfaces in rabbits.^{13–15} The BIV-infected rabbit model may have more value than the BIV-infected cattle model as an inexpensive small-animal model of HIV-AIDS.

CAPRINE ARTHRITIS–ENCEPHALITIS VIRUS (CAEV) AND MAEDI-VISNA VIRUS (MVV) INFECTIONS IN SHEEP AS MODELS

Both CAEV and MVV are transmitted in milk and by the exchange of body fluids, and the infections progress slowly.^{16–20} Unlike HIV infection in humans, CAEV and MVV infections in sheep do not involve CD4⁺ T cells, and neither secondary infections nor immunodeficiency is observed in the host. Younger sheep infected with CAEV develop neurologic signs of infection at 2 to 4 months of age, and those that survive exhibit persistent active inflammation in the central nervous system (CNS) and develop arthritis, mastitis, and encephalitis. Because of these characteristics, CAEV infection of sheep is considered a useful model for HIV-associated neurologic disease¹⁸ and for AIDS pneumopathies³ observed in humans.

Both MVV and CAEV are similar to HIV in that neutralizing antibodies resulting from the viral infection are ineffective for the viral clearance and lead to lifetime persistent viremia. Despite these similarities and those regarding viral infection and neurologic symptoms, sheep may have only limited use as practical models for laboratory-based AIDS research because these animals are rarely used in laboratories.

FELINE IMMUNODEFICIENCY VIRUS (FIV) INFECTION IN CATS AS A MODEL

FIV is the feline analogue of HIV. It causes severe impairment of the immune function in infected domestic cats, and the resulting disease closely resembles HIV-induced AIDS in humans. FIV and HIV are also structurally and biologically similar. Older cats infected with FIV exhibit decreased CD4⁺ T cell counts, inversion of the CD4:CD8 T cell ratio, and various clinical signs, including opportunistic infections.^{21–25} Disease progression in naturally FIV-infected cats, as in HIV-infected humans, starts with an initial acute stage characterized by fever, weight loss, decreased number of circulating neutrophils, and lymphoid hyperplasia that persists for several months. This stage is followed by a prolonged clinically asymptomatic period and the eventual development of immunodeficiency.^{26–27}

Immunohistochemical examination using FIV-specific antibodies from the serum of chronically infected cats identified T lymphocytes, macrophages, and dendritic cells (DC) as FIV antigen-bearing cells.²⁸ Thus, active replication in T cells and the capacity to infect long-lived reservoir populations (e.g., macrophages and DC) are properties common between FIV infection in cats and HIV infection in humans. In cats, as in humans, productively infected cells are identified in the bone marrow, lymph nodes, thymus, mucosal-associated lymphoid tissue, and spleen.²⁷ Mother-to-offspring transmission of FIV, as determined by detecting viral DNA and proteins in fetal tissues of cats by *in situ* hybridization and immunohistochemistry, has been documented;²³ therefore, the FIV-infected cat is considered a model for vertical HIV-1 transmission.

All of these properties argue strongly that the FIV-infected cat is a good model for HIV infection in humans. However, FIV and HIV infection also differ in important ways.^{24–26} Infection by FIV is mediated by host cell CD9 instead of CD4 as in HIV-1 infection. Another important difference is that only naturally infected cats, but not those experimentally challenged, will develop immunodeficiency, probably because concurrent infections by other agents, naturally found in domestic cats but not those used in the artificial laboratory environment, can serve as cofactors for pathogenicity.^{24–26} Even though experimental FIV infection of cats is not representative of HIV-mediated

immunosuppressive disease, the FIV-infected cat is still a good model for evaluating neurologic disease in humans with AIDS.

The FIV-infected cat may also be a good model for HIV vaccine development. Vaccination of cats with an attenuated strain of FIV resulted in protection against a highly virulent challenge with a heterologous FIV isolate.²⁷ This result has prompted interest in attenuated-HIV as a vaccine approach in humans.

HIV INFECTION IN SMALL ANIMAL MODELS

Transgenic Mice and Rats

The development of small-animal models for HIV infection, related pathology, and HIV-induced immunodeficiency is desirable for the simple reasons of low cost and ease of manipulation for developing intervention strategies. However, several hurdles related to species-specific barriers for viral entry and subsequent steps required for productive infection have precluded development of a single small-animal model that mimics all aspects of HIV infection and pathology. Therefore, a number of strategies have been adopted to translate different aspects of HIV-AIDS. These strategies include using transgenic mice expressing various receptors, coreceptors, and other accessory molecules of human origin necessary for viral entry and infection in a range of tissues and cell types²⁹ and using transgenic mice and rats expressing the full-length HIV genome or with mutations in viral sequences that silence certain functional activities to mimic various aspects of HIV-associated pathology and immunodeficiency.^{30,31}

Some of the finer details of HIV-1 entry into host cells became evident in studies with transgenic mice and rats that expressed the primary receptor and coreceptors for HIV. Productive infection occurs in rats but not mice because the HIV-1 *tat* gene is incompatible with cyclins in mouse cells but compatible with those in rat cells.^{32,33} Murine NIH 3T3 cells expressing *CD4*, *CCR5*, and *cyclin T1* genes of human origin were permissive for infection by the macrophage-tropic HIV strains (which require the CCR5 coreceptor), but production of progeny virus from infected cells was greatly reduced compared to human cells indicating major hurdles for viral assembly in nonhuman cells.³⁴

In a transgenic model where the HIV-1 promoter was used to drive the expression of the full-length HIV-1 provirus with the activities of the *gag* and *pol* regions functionally deleted, the transgenic rats showed viral gene expression (both structural and regulatory sequences) in various tissues and cell types and also displayed clinical symptoms reminiscent of HIV-AIDS.^{30,31} The disease symptoms included muscular wasting, skin lesions, neuronal cell loss, and selective type 1 helper T cell (TH1) immune abnormalities such as deficiency in delayed-type hypersensitivity to standard antigens and loss of T cells in lymph nodes.^{30,31} In light of the substantial similarities in the clinical symptoms of transgenic rats and humans with HIV-AIDS, this model should be useful for investigating therapeutic strategies against HIV-1 infection. Another important attribute of the transgenic rat model is its high-level viral gene expression in multiple tissues mediated by the viral promoter. In contrast, in a similar transgenic mouse model with a heterologous promoter, viral gene expression was restricted mostly to skin tissue because of a deficiency in the activity of *tat*, a positive regulator of HIV gene expression.³² However, in another transgenic mouse model, in which expression of the complete HIV coding sequences was under the control of regulatory sequences of the human *CD4* gene, the mice showed HIV gene expression in several T cell subsets and developed AIDS-like disease.³⁵ Modifications of this model by mutations introduced for selective deletion of various HIV genes revealed the importance of the HIV-1 *nef* gene in HIV pathology.³⁵ Thus, certain aspects of disease related to HIV gene expression can be studied in these transgenic mouse/rat models even though the natural course of HIV infection and AIDS cannot be truly replicated in these models.

The mechanism of reactivation of latent HIV has been studied using a line of transgenic mice that carried a defective HIV-1 genome.³⁶ Although the HIV-1 genome in the lymphocytes remained dormant under normal physiologic conditions, it could be reactivated by administration of lipopolysaccharide via induction of interleukin 1 (IL-1) alpha/beta and tumor necrosis factor alpha. This model was useful to determine the role of CpG methylation in HIV-1 latency because treatment of lymphocytes from these transgenic mice with the demethylating agent 5'-azacytidine resulted in demethylation of the *CREB/ATF* sites in the HIV-1 LTR, coinciding with reactivation of latent HIV-1 genome.³⁶

SCID Mice

The SCID mice lack functional T lymphocytes and B lymphocytes and therefore could be reconstituted with human cells or tissues for direct infection by HIV-1. However, HIV-1 infection of SCID mice with transplanted human peripheral blood lymphocytes (SCID-hu-PBL) or tissues (SCID-hu) does not result in AIDS-like disease. Nevertheless, some insights into HIV pathogenesis have been generated from studies using the SCID mouse model (reviewed in Reference 37). For example, SCID mice injected with HIV-1-infected monocyte-derived macrophages (MDM) into the basal ganglia developed behavioral and cognitive abnormalities associated with neuronal dysfunction and experienced decreased synaptic density similar to the encephalitis observed in humans infected with HIV-1.^{38,39} SCID mice are also useful in studies of HIV-specific protective immunity. Reconstitution of SCID mice with human T cells followed by injection of syngeneic HIV-1-infected MDM into the brain primed systemic antiviral CD8⁺ cytotoxic T lymphocytes (CTL) that migrated to the sites of the human MDM and caused their destruction.³⁹ Importantly, treatment of HIV-1 infection in SCID-hu mice with anti-inflammatory compounds such as platelet-activating factor [PAF] antagonist, tumor necrosis factor [TNF]-alpha release inhibitor, or highly active antiretroviral therapy (HAART) reduced brain inflammation, neuronal injury, and viral load in infected brain tissue.³⁹ Together, these results support the use of the SCID mouse model for studying the neuro-pathogenesis of HIV-1 infection and for testing novel therapeutic and vaccine strategies.

HIV, SIV AND SHIV INFECTIONS IN NONHUMAN PRIMATE MODELS

Chimpanzees (*Pan troglodytes*)

Soon after HIV was identified as the etiological agent of AIDS in humans, chimpanzees were recognized as one of the few living species susceptible to persistent HIV-1 infection. HIV infection in chimpanzees is characterized by persistent but low-level viremia and a concurrent high-titer antibody response. In chimpanzees, unlike humans, HIV infection of T cells but not cells of the monocyte/macrophage lineage occurs, and virus is not detected in the cerebrospinal fluid or brain.⁴⁰ Infection can persist up to 8 years without the loss of CD4⁺ T cells or the onset of immunodeficiency.⁴¹

Despite their large size, HIV-infected chimpanzees were enthusiastically pursued as potential model for developing vaccine and therapeutic strategies applicable to HIV-AIDS in humans because of their close phylogenetic relationship to humans and the ease with which primary patient HIV isolates could be introduced for productive infection. However, it quickly became evident that chimpanzees are relatively resistant to the development of AIDS, and injecting autologous infected cells, cell-free virus, or whole blood transfusions fails to initiate the onset or progression of disease.⁴¹⁻⁴⁴

Another obstacle to using HIV-infected chimpanzees as a model for AIDS is that they are very expensive and therefore, use of the required numbers of animals for obtaining statistically significant results is financially formidable. Thus, although chimpanzees are the only nonhuman primate

species that can be directly and productively infected with HIV-1, their usefulness as an animal model of HIV-AIDS in humans is limited. On the other hand, lack of disease in infected chimpanzees is a compelling reason for pursuing studies to understand the genetic and immune mechanisms of restricted viral replication and prevention of disease progression.

Baboons (*Papio cynocephalus*)

Like chimpanzees, baboons are closely related to humans genetically, anatomically, and physiologically and can be a model for studying HIV-AIDS and other human infectious diseases as well as for developing appropriate vaccine strategies.⁴⁵ Baboons are susceptible to infection by HIV-2. Similar to HIV-1-infected humans, HIV-2 infected baboons exhibit chronic infection with slow disease progression⁴⁵ and are therefore a nearly ideal model for AIDS caused by HIV-2, particularly for investigating the immunobiology of viral latency, clinical stages of disease, viral infection of lymphatic tissue, and viral transmission.^{46,47}

The control of HIV-2 infection in baboons, as of HIV-1 infection in humans, depends on both the cytotoxic and noncytotoxic antiviral activities of CD8⁺ T cells.^{46,47} Even though the large size of baboons can be viewed as a practical problem for their use as laboratory animals, compared to most monkey species baboons offer larger blood samples, providing many peripheral blood mononuclear cells for studying cellular immune responses relevant to viral control.⁴⁸ Furthermore, because baboons are not naturally infected with herpes virus B, handling of the animals and the blood products subsequent to infection with HIV-2 is relatively safe for animal care and laboratory workers.⁴⁹

Baboons have been shown to be susceptible to infection by four different HIV-2 isolates recovered from patients in West Africa with AIDS.⁴⁵ Serial animal-to-animal passage of the HIV-2_{uc2} isolate using whole-blood and bone marrow cells collected during the acute phase of infection resulted in a highly replicative and virulent isolate that quickly led to disease development.⁴ The baboon model has been used to study DNA vaccine strategies using novel cationic liposome formulations along with granulocyte macrophage-colony stimulating factor and the B7-2 molecule as genetic adjuvants.⁴⁷ Additionally, the pathogenic SHIV_{KU2} and SHIV_{89.6P} strains exhibited productive infection of microglial cells in baboons, and treatment with soluble CD4 and monoclonal antibodies to CCR5 and CXCR4 effectively inhibited viral infection.⁵ These results support the utility of the baboon as an alternative nonhuman primate model to study HIV-associated neuro-pathogenesis.

Monkeys

Of all the animal models explored for studies related to HIV-AIDS in humans, the SIV-infected monkey model has proven the most useful for understanding various aspects of HIV pathobiology and immunology. After infection with SIV, monkeys develop clinical symptoms similar to those seen in HIV-infected humans.

In the wild, the natural hosts for SIV are African monkeys: African green monkeys (*Cercopithecus aethiops*), sooty mangabeys (*Cercopithecus torquatus atys*), and African vervet monkeys (*Cercopithecus pygerrhus*). The three main SIV strains identified in these natural hosts are SIVagm, SIVsmm, and SIVcpz, respectively. On the basis of sequence homology, SIV can be divided into three groups: SIVagm, HIV-2/SIVsmm/mac, and HIV-2/SIVcpz.⁵ SIVsmm may be the progenitor of HIV-2 and SIVmac, which may have arisen due to cross-species transmission to humans and monkeys, respectively.^{5,50} None of the natural host monkeys exhibits signs of pathogenicity as a result of infection by any of these SIV strains, but disease virtually identical to AIDS induced by HIV-1 infection in humans appears after natural or experimental transmission to Asian monkey species, such as rhesus macaques (*Macaca mulatta*) and pig-tailed macaques (*Macaca nemestrina*).⁵¹ Therefore, the SIV-infected monkey model is frequently used for AIDS vaccine studies.

The next sections describe studies related to SIV infection in the natural host monkey species, and its functional similarities with and differences from HIV-1 infection, focusing on the utility of the SIV-infected monkey models in understanding HIV-associated pathology and in developing HIV vaccines.

Comparisons between the Primate Immunodeficiency Viruses and HIV in Terms of Infections in Respective Host Species

Natural host species that resist SIV infection are a unique resource for understanding the mechanisms underlying disease development and disease resistance. Thus, several laboratories have focused on inducing AIDS-like disease in different nonhuman primate species to elucidate mechanisms of HIV- or SIV-induced disease.

The hallmark of pathogenicity for both HIV and SIV infections is generalized immune suppression due to loss of and functional impairment of CD4⁺ T cells. Although SIV was predicted to be directly responsible for the loss of CD4⁺ T cells, the immune systems of sooty mangabeys remained functionally intact, possibly because of highly regulated homeostasis.⁵² Thus, the mangabey's naïve T cell compartment is preserved and intact after SIV infection, unlike that of HIV-infected humans, in whom T cells are progressively destroyed.⁵² HIV-induced AIDS may be a combination of virus-mediated direct T cell killing and chronic immune activation that eventually results in immune suppression or exhaustion, whereas natural SIV infection of monkeys in the wild could represent a perfect relationship between the host and virus that has evolved over time and is regulated by the host's immune system.^{50,51}

Functional Similarities

The early stages of both SIV infection in Asian macaques and HIV infection in humans are characterized by fever, skin rash, diarrhea, lymphadenopathy, viremia, neurologic symptoms, increased numbers of CD8⁺ T cells, and decreased numbers of CD4⁺ T cells.^{52,53} Despite the resulting large numbers of infected cells in lymph nodes, strong neutralizing antibody and CTL responses provide control of viremia in the early stage of both SIV and HIV infections.⁵⁴ Similar to HIV-infected humans with long-term nonprogressive disease, SIV-infected rhesus macaques with strong antiviral immune responses in the first 3 months after infection exhibit a long asymptomatic course and therefore are also classified as long-term nonprogressors.⁵⁴ Conversely, in both cases, weak antiviral immunity was associated with high viremia, rapid progression to AIDS, and death.⁵⁴ Furthermore, the disease course is variable in both humans and macaques, and polymorphic genes of the major histocompatibility complex play important roles in the initiation and regulation of antiviral cellular immune responses.

Functional Differences

The common mode of SIV transmission in monkeys is through the exchange of saliva and blood, during biting and fighting.⁵⁴ Sexual transmission, the major aspect of the human HIV-AIDS epidemic, has not been conclusively shown as a potential means of infection in monkeys, and the rate of mother-to-offspring transmission of infection appears much lower in monkeys than in humans.⁵⁴ The time to disease development after virus infection is generally 1 to 2 years in monkeys and 10 to 11 years in humans. Although the shorter time for disease development can be considered beneficial for quickly understand the immune correlates for protection or failure, such information in this artificially compressed disease course in SIV-infected macaques has to be carefully translated to humans for managing HIV infection or designing intervention strategies. Nonhuman primates rarely exhibit arthritis, antilymphocyte antibodies, skin rash, arteriopathy, Kaposi's sarcoma, or

vacuolar myelopathy, but these signs and symptoms are common in humans with HIV-induced AIDS.⁵⁴

Nonhuman Primate Models for Studies Related to HIV-Associated Pathology

The hallmark of HIV infection is a progressive decline in the number of CD4⁺ T cells accompanied by abnormalities related to hematopoietic and hematologic parameters and peripheral cytopenia.⁵⁵ Much of the current knowledge about these aspects of HIV pathogenesis in humans came from studies involving experimental infection of monkeys with SIV. In addition, a number of reports have described the use of the chimeric virus SHIV because SHIV infection in different nonhuman primate species results in a profound state of immunodeficiency characterized by severe loss of peripheral CD4⁺ T cells.^{56–59} The kinetics of lymphocyte loss differs depending on the pathogenic and nonpathogenic nature of the SHIV strains used.^{60,61} For example, infection of rhesus macaques with the nonpathogenic SHIV_{HXBc2}, which expresses the *env* gene of a T cell-tropic HIV-1, results in persistent infection without a decline in CD4⁺ T cell numbers, and the animals remain healthy for several years after infection.^{58,62,63} Similarly, infection of macaques with the nonpathogenic SHIV_{89.6}, which expresses the *env* gene of the primary HIV-1 isolate HIV-1_{89.6}, results in only a transient loss of CD4⁺ T cells, and despite persistent infection, the animals remain healthy for several years.^{58,63} However, in studies involving infection by SHIV_{KU2} and SHIV_{89.6p}, which were derived by serial *in vivo* passages of SHIV_{HXBc2} and SHIV_{89.6}, respectively, rapid and profound CD4⁺ T cell loss, opportunistic infections, and AIDS-like disease occurred.⁵⁸ Similarly, the molecular clone SHIV_{KB9}, derived from the animal used for the fourth passage of SHIV_{89.6}, was highly pathogenic, causing rapid loss of CD4⁺ T cells and mortality due to AIDS-like disease.⁶⁴

The importance of the HIV-1 accessory genes *nef* and *vpu* in the pathogenesis of HIV-1 was demonstrated in a study showing that corresponding sequences in SIV were responsible for SIV-mediated pathogenicity in certain rhesus macaques.⁶⁵ The abilities of *nef* and *vpu* in macaques were examined by serial passage of two nonpathogenic SHIV strains: SHIV-PPC and Δ *vpu**nef* SHIV-PPC. Animals infected with Δ *vpu**nef* SHIV-PPC maintained a limited phase of productive replication with no loss of CD4⁺ T cells, whereas animals infected with SHIV-PPC showed progressively enhanced pathogenicity in later passages, as evidenced by plasma viral load, viral mRNA in lymph nodes, infectious peripheral blood mononuclear cells, and loss of CD4⁺ T cells. This transformation coincided with the numerous mutations in SHIV-PPC, suggesting similar involvement of the *nef* and *vpu* genes and the LTR of HIV for the pathogenesis in humans.⁶⁵ This study clearly demonstrated the usefulness of the SIV-infected macaque model for HIV-associated pathology in humans.

Observations of HIV-infected humans suggest that the neurologic disease associated with HIV infection is related to the virus strain that uses CCR5 as the coreceptor or opportunistic infections in the CNS. Infection by SHIV_{KU2} in rhesus macaques but not pig-tailed macaques resulted in productive viral replication in the CNS and brain even though both species of macaques developed AIDS resulting in death.⁶⁶ Analysis of brain tissue from infected rhesus macaques revealed that virus replication in the brains depended on coinfection in the brain with opportunistic pathogens (e.g., *Schistosoma mansoni*) that presumably induced secretion of IL-4 in the CNS. Further, a supportive role for IL-4 in CNS disease was suggested by the presence of IL-4 RNA in the encephalitic brains of rhesus macaques and by reduced levels of this cytokine in the brains of pig-tailed macaques.⁶⁶ AIDS-related cognitive and motor function impairments are common in HIV-infected humans. These impairments were best illustrated in pig-tailed macaques coinoculated with SIV strains SIV/17E-Fr and SIV/DeltaB670, which accelerated CNS disease (SIV-encephalitis) in more than 90% of animals within 3 months.⁶⁷ These results from SIV-infected macaques showing a relationship between axonal damage and behavioral impairment strongly imply a role for HIV in the cognitive and behavioral abnormalities observed in patients with AIDS.

Nonhuman Primate Models for HIV Vaccine Development

Examples of successful viral vaccine strategies are the use of live attenuated virus for measles, inactivated wild-type virus for polio, and viral subunit for hepatitis B. All these vaccines stimulate long-lasting protective immunity in the host.⁶⁸ All of these approaches have been investigated for developing vaccines against HIV, but in nonhuman primate models, these approaches have not resulted in long-lasting immunity.¹ However, other vaccine strategies tested in nonhuman primate models have displayed variable levels of effectiveness against SIV or SHIV infection.

Inactivated Virus as an HIV Vaccination Strategy

Prompted by the proven effectiveness of inactivated virus as a vaccine for polio, a similar strategy was investigated for developing an HIV vaccine in the SIV-infected rhesus macaque model. Immunization of macaques with detergent-inactivated SIV_{mac} or formalin-fixed SIV was effective in preventing infection, but detailed analysis of immune correlates for protection revealed that immune responses specific to the human leukocyte antigens present as a contaminant in the vaccine preparation and in the challenge virus, rather than virus-specific immunity, were responsible for the observed protection.^{69,70} This finding combined with the general apprehension about the use of HIV in a vaccine, even after inactivation, has generally dampened enthusiasm for this vaccination strategy for HIV.

Passive Immunization as an HIV Vaccination Strategy

Compared with cellular immune responses that are constrained because of their dependence on the host's major histocompatibility complex molecules, antiviral neutralizing antibodies can be protective even when used for passive transfer between individuals and may be, in conjunction with appropriate immunomodulatory treatments, between closely related species. Several attempts were made in nonhuman primate models (macaques and chimpanzees) to test the protective efficacy of passively transferred anti-HIV antibodies isolated from infected individuals or induced in animals after experimental vaccination.^{1,71,72} One of the successful strategies within the small window between antibody infusion and infection is the use of a combination of monoclonal antibodies that have been well characterized for their *in vitro* virus-neutralization properties, to protect rhesus macaques against pathogenic SHIV infection.^{73,74} Similar passive transfer studies in pig-tailed macaques showed sterilizing protection by high-titer antiviral neutralizing antibody.⁷⁵ In general, the ability of a passive transfer approach using neutralized antiviral antibodies that effectively conferred sterilizing immunity was observed only when the timing of antibody infusion was close to experimental infection but not several hours or longer after infection.⁷²

Attenuated Virus as an HIV Vaccination Strategy

The attenuated virus vaccine is an attractive approach because deleting viral sequences critical for pathogenicity should be safe and because immunization with such a preparation should be effective in priming both humoral and cellular immune responses. Macaques vaccinated with a *nef*-deleted nonpathogenic strain of SIV_{mac} developed strong antiviral CD4⁺ T cell responses concurrent with expansion of mature DC and chemokine and cytokine production that is associated with protection against challenge with virulent SIV_{mac}.^{76–78} Similarly, using SHIV attenuated by deletion of the viral accessory genes *vpu* and *nef* and delivered by either parenteral (intravenous) or mucosal (intranasal and intravaginal) routes effectively primed the protective immunity in rhesus macaques against pathogenic strains of SHIV (SHIV_{KU2} and SHIV_{89,6p}) as well as SIV (SIV_{mac239}) after intravaginal challenge.⁶⁵ This protection was attributed to higher frequencies of SIV *gag*-specific CTL and IFN- γ secreting cells and an increase in IFN- γ mRNA levels in peripheral blood mononuclear cells. However,

immunization of infant monkeys by the oral route with an attenuated virus vaccine actually led to infection and mortality,⁷⁹ and prolonged follow-up of even adult monkeys immunized with attenuated vaccine revealed onset of disease.⁷⁸ Therefore, continued testing of attenuated vaccine strategies requires caution and detailed experimentation.

DNA Prime-and-Boost as an HIV Vaccination Strategy

In recent years, vaccination strategies involving primary immunization with DNA corresponding to selected antigens followed by boosting with the same antigens as soluble proteins or expressed from recombinant viral vectors have shown promise in generating protective immune responses against a number of infectious diseases and cancers. In the SIV-infected rhesus macaque model, using DNA-prime followed with soluble protein or recombinant modified vaccinia virus Ankara (MVA) expressing complete viral proteins as booster proved to be one of the best vaccination strategies for priming strong TH-1-polarized protective immune responses with a high frequency of CTL responses against diverse viral epitopes.⁸⁰ Other variations tested in the macaque model have included the use of vectors based on poxviruses, adenoviruses, lentiviruses, adeno-associated viruses, Sindbis viruses, and Venezuela equine encephalitis viruses^{81–93} for either priming or boosting. In all these cases, robust immunity and reasonable protection were demonstrated. However, therapeutic vaccination with recombinant vaccinia virus constructs in HIV-infected immunosuppressed humans with documented virus dissemination might be risky, because such vaccination can contribute to increased pathogenicity leading to morbidity and mortality.⁹³

In an effort to develop an effective AIDS vaccine that elicits a high frequency of antiviral CTL responses and is specific for a wide variety of viral epitopes, two prototype multiepitope plasmid DNA vaccines were tested in the SHIV-infected rhesus macaque model. The two vaccines demonstrated limited immunogenicity,^{81–84} but the same multiepitope construct inserted into an immunogenic SIV *gag* DNA vaccine elicited a higher frequency of CTL responses specific for all of the epitopes included in the vaccine.^{80–84} Further, boosting with recombinant MVA expressing the cognate viral proteins maintained the natural hierarchy of immunodominance for the individual CTL epitopes.^{80–84}

Because most HIV infections are acquired across mucosal surfaces, including the gut-associated lymphoid tissues, vaccine strategies designed to prime virus-specific mucosal CTL responses may be essential for protection. Based on this idea, a novel prime-and-boost strategy tested in a nonhuman primate model included three oral doses of recombinant strains of *Salmonella* expressing fragments of SIV *gag* fused to the type III-secreted protein SopE of *Salmonella* followed by a peripheral boost with MVA expressing SIV *gag*.⁹⁴ Even though control of virus replication after rectal challenge with SIV_{mac239} was not impressive, this study demonstrated the potential of mucosal priming by the *Salmonella* type III secretion system to direct SIV-specific cellular immune responses to the gastrointestinal mucosa in the primate model.⁹⁴ More recently, mucosal immunization by the intranasal route, in which SHIV DNA (alone or in combined with IL-2/immunoglobulin DNA or IL-2 DNA) was used for priming and recombinant MVA expressing SIV *gag-pol* and HIV_{89.6} *env* was used for boosting, was tested in macaques.⁹⁵ In this study, efficient mucosal immunoglobulin A (in rectal washes), systemic immunoglobulin G (in plasma), and cellular immune responses (in blood and lymph nodes) were observed in all the animals, whereas protection after rectal challenge with SHIV_{89.6P} was evident only in those animals that received the SHIV DNA plus IL-2/immunoglobulin DNA, indicating the need for and effectiveness of IL-2 for protection.⁹⁵

In yet another prime-and-boost vaccination strategy for protection against mucosal challenge, a replication-competent recombinant adenovirus expressing SIV *env/rev* along with *gag* or *nef* genes, followed by boosting with SIV gp120 or an SIV polypeptide mimicking the CD4-binding region of the envelope, was tested.⁹⁶ After rectal challenge with pathogenic SIV_{mac251}, anti-gp120 antibody production and cellular immune responses were observed and correlated with reductions

in virus loads at the acute phase and set point, respectively. These results demonstrate replication-competent recombinant adenoviruses as suitable and efficient vaccine delivery vehicles.

Several reports indicated the protective efficacy of antiviral CTL responses, but the role of antiviral antibodies in preventing or reducing infection was shown only by passive transfer studies.^{74,75} *In vitro* studies with antiviral antibodies from HIV-infected humans have been shown to enhance target cell infection, and the mechanisms suggested to explain this phenomenon implicated roles for complement and Fc receptors on human cells.^{97–99} An interesting finding related to the relative inefficiency for protection, and potential ill effects, of HIV envelope-specific antibody responses was observed in a test of a prime-and-boost strategy involving SHIV_{89.6} *gag-pol-env* DNA priming followed by boosting with SHIV_{89.6} *gag-pol-env* recombinant MVA alone or in combination with alum-adjuvanted HIV-1_{89.6} gp120.¹⁰⁰ Monkeys that received the booster dose containing gp120 raised binding antibodies, but not neutralizing antibodies. However, after rectal challenge with SHIV_{89.6p}, all animals failed to control virus, and those that received the booster containing gp120 exhibited infection-enhancing antibodies. Clearly discerning the *in vivo* role of the infection-enhancing antibodies may not be possible, but these results from the nonhuman primate model point to potential ill effects of nonneutralizing antiviral antibodies induced by HIV gp120.¹⁰⁰

Most of the prime-and-boost modalities tested for vaccination against HIV in the nonhuman primate model used HIV structural proteins. One study, however used a plasmid DNA encoding the HIV regulatory proteins *tat*, *rev*, and *nef* after removal of the nuclear localization sequence for Tat and Rev and the myristoylation site for Nef to avoid their known immunomodulatory activities.¹⁰¹ Priming with this DNA plasmid followed by boosting with the highly attenuated recombinant poxvirus vector encoding the same proteins was immunogenic in both SIV_{mac251}-infected macaques treated with antiretroviral therapy and treatment-naïve animals.

DC as Adjuvant for HIV Vaccines

The most potent antigen-presenting cells and the essential link between the innate and acquired immune responses, DC in the periphery can respond to invading pathogens through Toll-like receptors, acquire antigen, and migrate to draining lymph nodes to present processed antigens to the T cells. In a study of the potential usefulness of DC for prophylactic HIV vaccination, a highly conserved HIV-1 envelope peptide cocktail in the SHIV-infected rhesus macaque model was assessed.¹⁰² Macaques vaccinated with the peptide cocktail in Freund's adjuvant followed by autologous DC pulsed with these peptides exhibited strong antiviral cellular immune responses that effectively imparted protection against challenge with the pathogenic SHIV_{KU-2} and inhibited chronic infection and AIDS.¹⁰² The protection was through cell-mediated immunity, in particular TH1-type cytokine production, but it occurred in the absence of HIV-neutralizing antibody response because the vaccine peptide cocktail was composed of sequences that are specifically selected for priming cellular immune responses in the absence of antibody production. This study in the SHIV-rhesus model was unique in the sense that it enabled investigation of cellular immune responses selectively for protection.

The efficient APC function of DC was also harnessed for therapeutic vaccination in the SIV-infected rhesus macaque model. SIV-infected animals were protected by vaccination with DC pulsed with SIV that was chemically inactivated with aldrithiol-2, AT-2, resulting in a significantly lower number of SIV RNA copies than in control animals vaccinated with unloaded autologous DC.¹⁰³ This study used rhesus macaques of Chinese origin that do not exhibit SIV-mediated pathology, so the therapeutic efficacy of this approach is currently unknown.

Antisense Vector Vaccine

In rhesus macaques, an antisense vector vaccine reduced the pathogenicity associated with SIV infection.¹⁰⁴ In this approach, the vaccine was designed to target and degrade RNA sequences for

the SIV accessory proteins Tat and Rev, which are essential for productive infection. CD4 cells from the macaques were transfected with a retrovirus engineered to express the antisense Tat/Rev, which was injected into animals followed by SIV infection. Although all the vaccinated animals were infected, only limited SIV replication and disease progression were observed compared with control-infected monkeys. Further improvements of this strategy may yield vaccines with potential utility against HIV-AIDS in humans.

CONCLUSIONS

Efforts are underway worldwide to control the HIV-AIDS epidemic. Impressive progress has been made in developing therapeutic options, and well-organized attempts are under way to create prophylactic and therapeutic vaccine candidates against HIV and AIDS. However, the complexities of HIV infection in humans in terms of genetic susceptibility, antiviral immunity, and pathophysiology are enormous and cannot be easily and quickly resolved without systematic studies in suitable animal models. A major concern is the lack of an ideal model to address all these questions together. In addition, the ability of HIV to consistently exhibit productive infection only in humans, and to a lesser extent in chimpanzees, is problematic. Therefore, several animal models are currently used to investigate various aspects of HIV-1 infection and pathology. These attempts include testing other lentivirus family members (e.g., EIAV, BIV, CAEV, MVV, FIV, and SIV) that mimic features of HIV in terms of infection, host range, tissue tropism, and pathologic consequences. Different species are used for direct infection with HIV (e.g., chimpanzees, baboons, and transgenic rats and mice). Thus far, SIV and SHIV infections in rhesus macaques are the best-characterized models and have yielded useful information related to HIV-induced pathogenesis, therapeutics, and vaccine strategies. Pig-tailed macaques and baboons are excellent models for HIV-2 infection. In this chapter, we have compiled information available from various animal models used by different groups around the world to study different aspect of HIV infection and AIDS. In addition, we have summarized a few important HIV-1 vaccine strategies tested in nonhuman primate models.

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CHAPTER 5

Animal Models of Viral Diseases (Other than AIDS)

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INTRODUCTION

Knowledge based on the study of viral diseases in animals has contributed enormously to our understanding of how viruses infect, disseminate in, interact with, and damage the human host. It also laid the ground for advances in diagnosis, treatment, and prevention. Different levels of likeness of an animal model with a human disease may be distinguished. The appropriateness of the choice depends on the scientific question that is being addressed. Scientific projects that attempt to predict findings in animals for immediate application to humans require models that are close to the human disease under study, while studies that address more fundamental mechanisms can be permitted to have a larger distance. In some models the virus is the same, the disease induced is the same, and the species is very close to the human species: an example may be experimental hepatitis C virus infection in the chimpanzee. More often, however, dissimilarities exist such as that the virus differs, the species is phylogenetically distant from humans, or the disease induced is dissimilar to the one observed in the human host. One has to take these factors into account especially when extrapolating from an observation made in an animal model to humans. Other aspects not specifically dealt with include ethical considerations and those related to costs; both are especially relevant in studies involving larger animals, specifically primates. Below we discuss a selection of available animal models, focusing on their relevance to the study of human viral illnesses.

VIRAL INFECTIONS OF THE CENTRAL AND PERIPHERAL NERVOUS SYSTEM

In the United States, the annual number of central nervous system (CNS) infections that occur as a result of viral agents far exceeds that of infections caused by bacteria, yeast, molds, and protozoa combined.¹ Viral infections of the CNS can be rapidly progressive, producing permanent damage or death within a short period of time, or they can be characterized by persistence of the virus in its host. Widespread infections of the brain can be caused by viruses derived from various viral families and genera. In the following section, we will discuss the most common viral infections of the central and peripheral nervous system and list the animal models of importance.

Herpesviridae, Herpesviruses

Herpes simplex viruses (HSV) I and II are among the most intensively studied DNA viruses, partly because of their ability to remain latent in the host for life. They serve as model viruses for the study of synaptic connections in the nervous system, usually analyzed in rats.² Herpes simplex encephalitis (HSE) is one of the most devastating of all HSV I infections, causing the majority of cases of sporadic fatal encephalitis. The mouse model for HSE is an established preclinical tool for evaluating the efficacy of new therapeutic interventions³ and vaccine development. Many animal models have been used in studies on viral latency including mice,⁴ guinea pigs,⁵ rats,⁶ and rabbits.⁷ The rabbit eye model is particularly useful for studies of spontaneous reactivation.⁸ Finally, a unique murine model based on SJL mice faithfully mimics human disease and might be very helpful for further analysis of HSE.⁹

Paramyxoviridae

Mumps virus is a negative-sense RNA virus classified as a member of the genus *Rubulavirus*, causing widespread infection, and due to its high neurotropic properties, frequently leading to aseptic meningitis or meningoencephalitis. Live attenuated mumps vaccines, available since the 1960s, and measles–mumps–rubella (MMR) vaccines drastically reduced the number of mumps cases reported. The only natural host is humans, but experimentally, mumps can be induced in the rabbit, guinea pig,¹⁰ adult and suckling hamster,^{11,12} suckling mouse,¹³ and rat,¹⁴ as well as in the monkey,¹⁵ ferret,¹⁶ and dog.¹⁷ The suckling hamster model is of particular interest, as it demonstrated that a “neuroadapted” mumps virus strain, originally isolated from breast milk, produced a severe meningoencephalitis in suckling hamsters after intracerebral inoculation.¹⁸ This model is used to explore viral genetic factors that contribute to neurovirulence;¹⁹ and neurovirulence tests in hamsters,²⁰ monkeys,²¹ and most successfully in rats²² are used to discriminate between neurovirulent and nonneurovirulent human mumps virus strains. At present, research mainly focuses on the evaluation of attenuated vaccines and possible related CNS complications analyzed in mice²³ or marmosets²⁴ and the development of new vaccine types.²⁵

Measles virus (MV) is an enveloped, negative-sense RNA virus belonging to the *Morbillivirus* genus, causing a typical childhood illness. CNS complications such as encephalitis occurring after acute measles infection are a serious problem. Naturally, MV infects only humans and nonhuman primates. Monkeys can either be infected through the respiratory route by humans or experimentally, and the disease mimics infection in humans.²⁶ Monkeys serve as a model for analysis of pathogenesis,²⁷ virus virulence,²⁸ and vaccine-induced protection from measles,²⁹ as well as factors responsible for protection.³⁰ Also susceptible to infection are tamarins and marmosets, but they develop different symptoms than humans do. Marmosets have been successfully used to identify virulence of virus strains in humans.³¹ Small laboratory animals generally are not susceptible to wild-type (wt) strains of MV, with the exception of limited replication of the virus in cotton rats.³² Repeated intracerebral passage has led to several neurotropic, rodent-adapted strains that are useful for the

study of the resulting neurologic disease³³ in newborn hamsters³⁴ and mice,³⁵ ferrets,³⁶ neonatal rats,³⁷ and cotton rats.³⁸

***Picornaviridae*, Enteroviruses**

Poliovirus (PV) is a positive-stranded RNA virus causing aseptic meningitis or paralytic poliomyelitis that results from the destruction of spinal cord anterior horn motor neurons, as a consequence of virus replication and apoptosis, which was determined in mice.³⁹ Humans are very susceptible to infection and seem to be the only natural host. The analysis of animal hosts revealed that PV is restricted to simians, including chimpanzees, as well as rhesus, cynomolgous, and African green monkeys, due to a rapid sequence change on the poliovirus receptor (PVR) gene during evolution.⁴⁰ Introduction of this PVR gene into mice results in transgenic (tg) animals sensitive towards poliovirus.⁴¹ Importantly, the relative levels of neurovirulence originally established to test oral poliovirus vaccines in a monkey model seem to correlate well with the results obtained in these mice,⁴² which are susceptible to infection after intraperitoneal, intracerebral, and intramuscular inoculation of the virus.⁴³ Therefore, many analyses for further vaccine development can be performed in mice instead of monkeys,⁴⁴ which is a plus for financial reasons. Furthermore, the tg mice develop similar signs of illness as humans do and can be easily used to analyze human poliomyelitis.⁴⁵ Also, there is an *ex vivo* murine model, which is used to analyze PV-induced apoptosis in nerve cells growing in cell culture⁴⁶ and which might partly replace the animal model.

***Arenaviridae*, Arenaviruses**

Lymphocytic choriomeningitis virus (LCMV) is a rodent-borne, negative-stranded RNA virus causing rarely fatal diseases in humans ranging from flu-like illness to meningitis or encephalitis.⁴⁷ It serves as the number one model virus for viral immunologists.⁴⁸ There is a reservoir of LCMV in mice, and when humans come in contact with excreted virus, disease may result. Experimentally, rodents — particularly mice (the natural host),⁴⁹ guinea pigs,⁵⁰ hamsters,⁵¹ and newborn rats⁵² — can be infected, with the outcome depending on strain, dose, route of infection, and host genotype. Murine infection with LCMV is illustrative of the balance between immunologic protection and immunopathology. Finally, macaques are a possible but not perfect model for the pathogenesis and experimental therapy of LCMV infections in humans, as the disease in this species resembles hemorrhagic fever rather than the CNS disease of humans.⁵³

***Rhabdoviridae*, Lyssaviruses**

Rabies virus is an enveloped, negative-stranded, highly neurotropic RNA virus belonging to the very diverse family *Rhabdoviridae*. It can potentially infect all warm-blooded animals — ranging from dogs to raccoons, foxes, skunks, and bats.⁵⁴ Rabies infection in humans is one of the most common causes of encephalitis. Studies of rabies pathogenesis began in 1804, when Zinke transmitted rabies by swabbing the saliva of a rabid dog onto some fresh wounds of a dachshund,⁵⁵ and were later continued by Pasteur, who routinely transmitted rabies from animal to animal.⁵⁶ Mice and rats are often used in experimental rabies, focusing on the spread of the virus to the CNS,⁵⁷ within the CNS (by fast axonal transport),⁵⁸ and from the CNS,⁵⁹ using various sites of inoculation. However, the strain,⁶⁰ the age, and the immune status of the host⁶¹ are very important factors for the outcome of an infection. The suckling mouse model is primarily used to analyze the budding of the virus from the neuronal cell membrane.⁶² Quite a few studies are also performed *in vitro*, mostly focusing on the infection and outcome in specific neuronal cell types.⁶³ Analysis of vaccine development is usually based on a variety of nonhuman primates such as rhesus monkeys,⁶⁴ capuchin monkeys,⁶⁵ bonnet monkeys,⁶⁶ and macaques⁶⁶ or on nondomestic hosts such as skunks⁶⁷ and raccoons.⁶⁸

***Bunyaviridae*, Bunyaviruses**

La Crosse virus (LACV), a member of the California serogroup of Bunyaviruses with a genome composed of three single-stranded RNA segments, is the major arboviral cause of pediatric encephalitis in the midwestern United States.⁶⁹ The virus is mostly transmitted by the mosquito *Aedes triseriatus*, and chipmunks and squirrels are natural hosts, whereas humans are dead-end hosts. Experimentally, foxes, raccoons, opossums, and woodchucks have been successfully infected.⁷⁰ LACV is a recently emerging zoonotic virus, and as only suckling mice are susceptible to it,⁷¹ Schuh et al. developed an artificial, sensitive animal model: genetically targeted mice lacking a functional interferon type I receptor (IFNAR-I), which were used in the very first DNA-based vaccine studies.⁷²

***Flaviviridae*, Flaviviruses**

St. Louis encephalitis virus (SLEV),⁷³ mostly transmitted via the mosquito *Culex pipiens*, and Japanese encephalitis virus (JEV),⁷⁴ transmitted by *Culex tritaeniorhynchus*, are among the most important arthropod-borne Flaviviruses causing encephalitis in humans in the United States and Asia, respectively. The main hosts are birds, aquatic birds as well as pigs for JEV, and humans are only incidentally infected from the enzootic cycle. SLE has been isolated from a variety of wild vertebrates including birds, raccoons, opossums, bats, rodents, and nonhuman primates. Infant mice and hamsters and young rats are highly susceptible to infection, whereas guinea pigs, older rats, and rabbits develop antibodies, but not disease. Mice have been repeatedly used for vaccine studies,⁷⁵ intrauterine infection,⁷⁶ or — together with hamsters⁷⁷ — as models for the analysis of the neurologic spread.⁷⁸ Monkeys can be used as a model to analyze the virulence of different viral strains.⁷⁹ Similar to SLEV infection, infant mice are also highly susceptible to JEV. Many studies based on vaccine development have been performed either in mice,⁸⁰ rabbits, or monkeys.⁸¹ In contrast to SLEV, several vaccines against Japanese encephalitis are currently available.⁸²

***Togaviridae*, Alphaviruses**

Alphaviruses are plus-stranded RNA viruses that are transmitted by mosquitoes and cause a broad range of diseases. Their principal vertebrate hosts are birds, rodents, and primates, although wallabies, equines, bats, and other animals may play a role, too. Rodent hosts, especially neonatal mice, which develop fatal encephalitis after intracranial (i.c.) inoculation of all strains, but also adult mice, hamsters, and guinea pigs, are frequently used for the isolation of alphaviruses as well as for the examination of pathogenic mechanisms.⁸³ Semliki Forest Virus (SFV) is the model virus to study pathogenic processes occurring in viral encephalitis.⁸³ Depending on the viral strain and the age and immunocompetence of the host, however, experimental infection of mice with SFV can vary greatly.⁸⁴ The neurogenic spread of the virus,⁸⁵ the role of B cells and antibodies⁸⁶ or CD8⁺ T cells⁸⁷ in the pathogenesis, and the breakdown of the blood–brain barrier have been extensively studied in mice,⁸⁸ which are also very popular for vaccine studies.⁸⁹ On the other hand, *in vitro* models based on rat primary cell cultures are used to analyze the virus growth of different strains.⁹⁰

Venezuelan Equine Encephalitis Virus (VEEV) is very infectious even by aerosol inhalation, and pathogenesis has been examined in experimental infections of horses,⁹¹ monkeys,⁹² bats,⁹³ wild mammals living in North America,⁹⁴ and most importantly rodents such as hamsters,⁹⁵ mice,⁹⁶ and rats.⁹⁷ While mice show paralysis, hamsters do not survive long enough to develop any neurological signs. Therefore, the mechanism of viral spread and neuroinvasion,⁹⁸ the protective effects of melatonin,⁹⁹ and the effects of recombinant vaccines against VEEV¹⁰⁰ have been mainly analyzed in mice.

VIRAL INFECTIONS OF THE LIVER

Viral hepatitis still represents a major public health problem in humans. Hepatitis viruses in humans (hepatitis A to E viruses) are predominantly hepatotropic and belong to at least five different virus families. Hepatitis A virus (HAV) and hepatitis E virus (HEV), both of which are RNA viruses, are enterically transmitted and associated with acute hepatitis, whereas hepatitis B virus (HBV), a pararetrovirus; hepatitis D virus (HDV), a circular RNA virus similar to plant satellite viruses; and hepatitis C virus (HCV), another RNA virus, are transmitted via the parenteral route. They are noncytopathic viruses similar to lymphocyte choriomeningitis virus (LCMV) in mice and able to persist in the host for years and to evolve to chronic hepatitis, often leading to development of hepatocellular carcinoma (HCC).

There remain a number of hepatitis cases in which no virus has thus far been detected. The recently identified hepatitis GB virus C (GBV-C), TT viruses (TTVs), TTV-like mini viruses (TLMVs), and SEN viruses (SENVs) commonly infect humans but do not appear to be associated with any disease.^{101–103} GBV-C, a very similar virus also formerly called hepatitis G virus (HGV), appears to primarily replicate in lymphocytes and not hepatocytes and to be associated with prolonged survival in human immunodeficiency virus–infected individuals. TTV, the genetic diversity of which greatly exceeds that of any other known virus, even that of papillomaviruses infecting the skin — the genomes of TTVs markedly differing in sequence and size — might be zoonotic, as human TTVs and TTV-like animal viruses are prevalent not only in many nonhuman primates and tupaia but even in humans themselves, as well as in farm animals such as sheep, goats, cows, pigs, and chickens and in cats and dogs. Chimpanzees and rhesus monkeys have been shown to be susceptible to TTV infection through either parenteral or oral inoculation. Viremia persisted and virus could be found in hepatocytes without obvious pathology, and virus was excreted in feces, making spread by enteric routes possible. Hepatitis F virus (HFV), originally recognized in the stool of a patient suffering from non A-E hepatitis, appeared to be a ubiquitous nonpathogenic intestinal resident virus.

Hepatitis A to E viruses show — with the exception of HAV that has been adapted to growth in continuous cell culture — substantial restriction for propagation in cell cultures. Animal models are therefore needed to study the virus life cycle, pathogenesis, and humoral and cellular immunity. Moreover, in recent years, the broad emergence of novel therapeutic agents directed against human liver disease, such as antivirals, small interfering RNAs (siRNAs), therapeutic vaccination, and human monoclonal antibodies, enhanced the need to further establish novel animal models to evaluate safety and efficacy of treatment prior to usage in humans. Human hepatitis viruses have a very narrow host range and are not infectious for immunologically well-defined laboratory animals such as mice. Only nonhuman primates, among them especially the chimpanzee, are susceptible to direct inoculation with human hepatitis viruses. In past decades, they played an important role in elucidating various aspects of host–viral interactions, including evaluation of safety and induction of protective immunity of prophylactic vaccines, and even led to the discovery of HCV. Nowadays, for ethical reasons, the research use of chimpanzees, which are endangered in the wild, is very restricted. Reliable small-animal models of viral infections, including surrogate models of nonhuman hepatitis viruses, and development of cell culture systems allowing efficient propagation of different viruses will provide the major tools in the near future in studying viral pathogenesis, including elucidation of still-unknown viral receptors and novel therapeutic agents.

Hepatitis B and D Animal Models

Among experimental animals, only the chimpanzee (*Pan troglodytes*), the primate-like tree shrew (*Tupaia belangeri*), and the baboon (*Papio ursinus orientalis*) have been shown to be susceptible to HBV infection.¹⁰⁴ In contrast to experimentally infected chimpanzees, which show only mild liver disease, thus allowing only study of the mechanisms of acute hepatitis and viral

clearance, infected tree shrews, in addition to transient viral hepatitis, may develop chronic hepatitis and HCC, similar to what is seen in humans, though at low incidence. Thus, this model can be used to study the efficacy of prophylactic HBV DNA immunization, as well as mechanisms of hepatocarcinogenesis. Baboons, which have been considered as a source of xenografts for human organ transplantation, do not show biochemical or histological evidence of liver disease upon viral inoculation, but viral DNA persists in serum and liver tissue for weeks. Use of xenografts from these animals should therefore be avoided. Recently, a small monkey originating from Morocco, *Macaca sylvanus*, has been successfully transfected intrahepatically with cloned HBV and experienced acute hepatitis; this could develop into a useful animal model for study of viral replication in the future.¹⁰⁵ Novel hepadnaviruses closely related to HBV have been isolated from a wide range of great and lesser apes as well as from New World monkeys (but not from Old World monkeys), among them woolly monkey HBV (WMHBV) isolated from the woolly monkey (*Lagothrix lagothricha*). Since woolly monkeys are also endangered, closely related spider monkeys (*Ateles geoffroyi*) have been used as an animal model instead and were successfully infected with WMHBV, but persistent infection has not been induced thus far.¹⁰⁶

Several nonprimate hepadnaviruses have been isolated from various species of avians and rodents, including the two best studied, the duck hepatitis B virus (DHBV), which infects white Pekin ducks (*Anas domestica*), and the woodchuck hepatitis virus (WHV), which infects eastern American woodchucks (*Marmota monax*).^{107,108} Ducks and woodchucks may be acutely or chronically infected with their respective viruses but, in contrast to humans, do not develop cirrhosis. Whereas chronic liver inflammation in ducks is usually mild and rarely progresses to HCC, in woodchucks, progressively severe hepatitis and HCC develop in virtually every chronically infected animal within a short period of time. In the past, the duck model has been very useful in characterization of molecular mechanisms of hepadnaviruses, e.g., their replication strategy and the role of covalently closed circular DNA (cccDNA) as the transcriptional template of the virus. Today, this model is mainly used for studies of viral kinetics, antiviral drug screening, and efficacy of therapeutic vaccination in chronically DHBV infected ducks, though one has always to take into account that ducks and DHBV are evolutionary remote from humans and mammalian HBVs, respectively, and prediction of toxic drug effects might not be possible. Similar to the avian model, the woodchuck model is broadly accepted for investigating novel antiviral agents and novel immunotherapies, including prophylactic and therapeutic vaccination. In addition, due to the close relatedness of WHV to HBV and the course of infection in infected individuals, pathogenesis and molecular mechanisms of chronic liver disease and HCC, as well as mechanisms of viral reinfection of the liver after transplantation and viral clearance and occult persistence and lymphotropism of hepadnaviral infection, have been extensively investigated in this animal model. However, as pathogenesis and clearance of HBV infection are believed to be immune mediated, the unavailability of inbred woodchucks and the paucity of woodchuck-specific cytokines and reagents to analyze their immune cells limit elucidation of the cellular basis of liver disease in this animal model.

For mice, inbred animals are available, but they are not susceptible to HBV infection. To overcome this disadvantage, in recent years, several transgenic mice have been developed that carry and express one or more HBV transgenes including transgenes that fully support viral replication showing high titers of infectious virions in respective sera.¹⁰⁹ However, viral cccDNA, as in the natural course of HBV infection, is not produced in these mice, and the animals are immunologically tolerant to the products of the transgenes, so no viral replicative life cycle can be studied and no liver disease is observed. Nevertheless, novel antiviral compounds can be evaluated in this noninfectious model, though sensitivity to drugs and kinetics of drugs cannot be compared to the situation in humans, as integrated DNA in mice is not influenced by antiviral treatment and mouse hepatocytes are not susceptible to new rounds of infection with virus. The pioneering work of Chisari et al. using transgenic mice models contributed substantially to the elucidation of noncytolytic mechanisms of immune control and clearance of viral liver disease, mainly by administration of various cytokines and adoptive transfer of educated immune cells to these mice.¹¹⁰

A humanized animal model of chronic HBV has been established by another group using double-transgenic mice expressing a chimeric HLA-A2 MHC class I molecule and high amounts of hepatitis B surface antigen (HBsAg) in hepatocytes.¹¹¹ Breaking of tolerance towards the surface antigen was observed upon DNA-based immunization when accompanied by unspecific immune stimulation.

In another transgenic mouse model of hepatitis B, it has been shown that expression levels of HBsAg, which is believed to be involved in maintenance of peripheral tolerance towards the protein, lower and progressive liver disease evolves with aging of the animals, and breaking of tolerance towards the antigen upon therapeutic vaccination was observed in older mice.¹¹² Recently, a transgenic mouse model has been developed with the aim to study HBV-associated chronic liver disease in the absence of tolerance against viral proteins. A transgenic mouse model supporting HBV replication has been made using severe combined immunodeficient (SCID) mice lacking mature B and T cells.¹¹³ Adoptive transfer of unprimed syngeneic splenocytes resulted in development of chronic hepatitis in these mice. Naïve immune responses can therefore be evaluated in this mouse model system.

HBV gene expression, replication, and liver disease have also been studied in a number of nontransgenic rodents. Attempts were made to induce viral hepatitis in rats by transfection of HBV DNA into rat livers or HBV-transfected liver tumor cell lines or nontumorigenic, immortalized primary human hepatocytes into nude mice or into mice that were deficient for recombination-activating gene-2 (Rag-2), respectively, with different degrees of success.¹¹⁴ The technique of *in vivo* transfection of naked HBV-DNA through acute circulatory overload (so-called hydrodynamic transfection) allowed for successful establishment of acute and long-term HBV infection models in immunocompetent and immunocompromised mice, with expression of HBV DNA primarily in the liver.¹¹⁵ HBV replication and new treatment options such as RNA interference can be studied easily in this novel small-animal model. Recently, various murine and rat chimeric liver models have been established, allowing for long-term engraftment of hepatocytes with natural infection of cells upon inoculation with hepatotropic viruses. In one such model, xenogenic hepatocytes from woodchucks and humans, respectively, have been successfully engrafted into livers of Rag-2-deficient mice that were in addition transgenic for a hepatotoxic protein (liver specific albumin promoter-driven urokinase-type plasminogen activator, *Alb-uPA*) (the SCID/*Alb-uPa* mice) via intrasplenic injection.¹¹⁶ These new small-animal models allow the study of all phases of viral hepatitis from natural infection of hepatocytes with production of cccDNA to development of HCC and evaluation of anti-HBV therapeutic agents.

In another small-animal model, immunocompetent rats were rendered tolerant to engrafted human hepatocytes by injection of cells at fetal age.¹¹⁷ The chimeric rats were susceptible to HBV infection after birth. Finally, an HBV-Trimera mouse model made up from three genetically disparate sources of tissue has been established as a tool for studying viral hepatitis B and for evaluating potential anti-HBV therapeutic agents including human poly- and monoclonal antibodies used to prevent reinfection of liver in transplanted patients and to reduce viral load in patients with chronic hepatitis B.¹¹⁸ In the Trimera system, animals were rendered immunoincompetent by lethal total body irradiation and radioprotected with bone marrow cells from SCID mice permissive for engraftment of functional human tissue fragments, which have been infected *ex vivo* — e.g., with hepatotropic viruses or blood cells.

In nature, HDV has only been found in humans. As HDV is a defective virus, it needs the co-presence of envelope protein of hepadnaviruses for its transmission/survival allowing experimental infection of chimpanzees, tupaia, eastern woodchucks, and Pekin ducks.¹¹⁹ Experimental transfection of HDV RNA has been also performed in mice, and mice transgenic for HDV sequences have been made in the past. Recently, hydrodynamic transfection of nontransgenic and HBV-transgenic mice with *in vitro*-transcribed RNA and/or HDV-encoding plasmids provided convenient mouse-based models of HDV infection capable of yielding viremia in the latter model to study prenylation inhibitors, a novel class of antiviral agents to prevent production of HDV virions, *in vivo*, as HDV

virion assembly is critically dependent on prenyl lipid modification, or prenylation, of large delta antigen, a nucleocapsidlike protein.¹²⁰ These novel animal models are technically less demanding than a previously established xenotransplantation model for HDV in immunodeficient nonobese diabetic/SCID mice, where xenogenic primary human hepatocytes susceptible to HBV infection and superinfection with HDV were ectopically transplanted beneath the kidney capsule.

Hepatitis C and GBV-B Animal Models

HCV has been molecularly cloned and identified as the major agent responsible for the disease that at one time was called non-A, non-B hepatitis. Over the past two decades, the chimpanzee model was the only animal model for research on HCV, which is naturally only found in humans, and for which, unlike HBV, no closely related animal viruses exist.^{104,121,122} Besides the chimpanzee, other animals including other nonhuman primates do not appear to be susceptible to HCV infection with the possible exception of rhesus monkeys (*Macaca mulatta*) and the primate-like tree shrew, which experienced transient hepatitis upon intravenous inoculation with infectious HCV containing serum after having been irradiated. In future, the chimpanzee model will continue to be an indispensable model for HCV research, especially for vaccine research. The chimpanzee is less useful to study HCV pathogenesis, as infected animals very often clear the virus and rarely develop chronic liver disease. A surrogate animal model for HCV research is the GB virus B (GBV-B)/tamarin (*Saguinus* species) model. GBV-B, an unclassified animal hepatotropic virus, is the closest relative to HCV and less related to the nonhepatotropic viruses GBV-C and GB virus A (GBV-A), which apparently lack a nucleocapsid coding region. In contrast to GBV-C-like viruses and GBV-A, which are naturally found in chimpanzees and many New World primates, respectively, without causing obvious liver disease, GBV-B causes acute hepatitis in New World monkeys with very high levels of viremia.¹²³ Recently, synthetic GBV-B RNA clones established chronic hepatitis in tamarins upon intrahepatic inoculation of animals with viral RNA, allowing for analysis *in vivo* of HCV replication and for evaluation of candidate antivirals in a small-primate model in the near future.¹²⁴

Several transgenic mouse models of HCV pathogenesis have been established in recent years, with variable results with respect to observed liver disease.¹²⁵ Nevertheless, they allow study of the pathology of specific HCV gene expression, as well as analysis of cellular and humoral immune responses upon genetic immunization. Very recently, a transgenic mouse model has been developed that allows for conditional expression of the HCV genome 2 months after birth of the animals, with subsequent development of liver inflammation, resembling the events observed in natural infection in humans.¹²⁶ Similarly to HBV, a SCID/*Alb-uPA* and a Trimer mouse model have been established for HCV, allowing for testing of potential novel therapeutic agents against the virus.^{118,127} A different approach to study HCV was made by establishing a model based upon *ex vivo* infection of orthotopically implanted HCC cells in athymic nude mice.¹²⁸ Viral replication including viremia did occur in this mouse model. Hydrodynamic transfection of HCV RNA in a mouse model allowed translation of transfected RNA, but viral replication did not occur.¹²⁹ Nevertheless this mouse system is suitable for assessment of novel antivirals, such as HCV translational inhibitors, *in vivo*. Similarly to HBV, a small-animal model for HCV was developed in immunocompetent rats that were tolerized to Huh-7 cells at fetal age and inoculated with human serum containing viral RNA from genotype 1b after birth.¹³⁰ HCV replication could be demonstrated in the livers of the animals.

A major breakthrough in HCV research was the development of infectious cDNA clones of HCV.¹²⁵ RNA transcribed from cloned HCV cDNA was found to be infectious and to cause liver disease in chimpanzees after direct intrahepatic inoculation. Infectious synthetic RNA provides an excellent tool for the study of viral gene structure and function and replication of the virus, as well as host tropism and pathogenesis of HCV.

Recently, a promising research model has been developed for molecular studies of HCV replication: the replicon (HCV-minigenome) model system.¹³¹ Both subgenomic replicons lacking genes encoding structural proteins as well as full genome-length HCV replicons allow replication

of HCV virus in cell culture and analysis of encoded proteins. Until recently, for unknown reasons, only a particular genotype of HCV (genotype 1b) succeeded in replicating in one particular cell line of hepatic origin (Huh-7), and infectious particles were never detected. HCV replicons with adaptive mutations have subsequently been established in a human cell line of nonhepatic origin (HeLa) and even in a nonhuman cell line from mouse liver.¹³² Recently, inoculation of human liver cells grown in a three-dimensional radial-flow bioreactor with infectious human serum resulted not only in viral replication but also in production and release of HCV virions into the supernatant.¹³³

Hepatitis A and E Animal Models

HAV and HEV are globally distributed in humans and widespread also in animals (for a recent review see Purcell and Emerson and references therein).¹³⁴ Antibody prevalence studies revealed naturally occurring infections with HAV in various nonhuman primates, leading to the discovery of simian strains of HAV recovered from cynomolgus monkeys and one vervet. Owl monkeys appeared to be naturally infected with a human HAV strain. The natural history and pathogenesis of replication-competent infectious virus can be assessed in animal models through successful transmission of HAV to certain species of nonhuman primates, mostly chimpanzees and tamarins (*Saguinus* sp.), but also owl monkeys (*Aotus trivirgatus*) and marmosets (*Callithrix* sp.), but not to small laboratory animals. *In vivo* intrahepatic cDNA transfection has also been performed in nonhuman primates. Nonhuman primates are rarely symptomatic during experimental HAV infection and all recover completely; nevertheless, they proved to be suitable for studying viral pathogenesis and also for testing of prophylactic vaccines.

A nonprimate animal model for HAV is still warranted. As it is known that HAV adapted to tissue culture can grow in guinea pig cell cultures, experimental infection of guinea pigs with HAV was performed.¹³⁵ Fecal shedding of HAV could be demonstrated upon experimental HAV infection of the animals, but no clinical or biochemical liver disease appeared and no antibodies to the virus were produced, perhaps due to the low infectious titer of the inoculum used in the experiments. Further studies will show whether an alternative nonprimate rodent small-animal model can be established for pathogenesis of HAV.

HEV may be much more prevalent in industrialized countries than previously appreciated. Except for pregnant women in some developing countries, in whom often-fatal fulminant hepatitis does occur, infection with HEV is usually not life threatening. In addition to swine, rodents, and chickens, from which swine HEVs, which cluster phylogenetically with a human HEV isolate, and murine and chicken HEV-like viruses, respectively, have been isolated, there is serological evidence of widespread infection of HEV among wild rats, cattle, deer, dogs, and various monkeys.¹³⁶ Thus HEV reflects a zoonosis, and cross-species infection is likely to occur, as swine can be experimentally inoculated with swine HEV and human HEV as well, with pigs and possibly rats and deer serving as reservoirs for the human disease. Foodborne HEV infection may also be possible.¹³⁷ In addition, swine HEV in pig organs or tissues might be transmitted to humans through xenotransplantation.¹³⁸ Experimental transmission with HEV to various animals, including pigs and rats, has been reported. Until now, rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fascicularis*) macaques have been the most useful models for study of the natural history of the virus. Pigs and rats might provide alternative animal models in the near future. Usually, viruses are administered intravenously to obtain clinical signs of acute hepatitis E, not orally as in the natural course of the disease. Recently, synthetic RNA was used to initiate an infection in chimpanzees and rhesus monkeys by intrahepatic inoculation.

The nonhuman primate animal models for HEV have provided a useful method for elucidation of various aspects of HEV pathogenesis, as well as for evaluation of candidate prophylactic HEV vaccines. Several vaccination studies with recombinant protein, viral-like particles, and naked DNA

have been performed in primates, showing protection of vaccinated animals against infection, and initial studies in humans are currently under evaluation.^{139,140}

VIRAL MYOCARDITIS

Even though some cardiotropic viruses such as parvovirus B19, adenoviruses, cytomegalovirus, and less frequently Epstein–Barr virus could be detected by endomyocardial biopsy, two major viruses have been extensively associated with myocarditis: coxsackievirus B3 (CVB3), a positive-sense RNA virus and member of the *Picornaviridae* and the Enterovirus genus; and reovirus, a dsRNA virus that belongs to the *Reoviridae* and the Reovirus genus. Viral myocarditis is a major concern in our society – in fact, it is estimated that ~5% of any virus-infected population experience some cardiovascular symptoms, with higher proportions during epidemics in late summer. Also, it is generally well accepted that one-third of patients with viral myocarditis experience a complete recovery of normal cardiac function, one third improve clinically but show residual cardiac dysfunction, and one third experience chronic heart failure and require heart transplantation or die.¹⁴¹ Due to the severity and high frequency of viral myocarditis, many research groups worldwide work in this field.

Picornaviridae, Enteroviruses

Enterovirus infections are extremely common, and coxsackieviruses B3, A4, A14, A16, B1, B5, and others have been associated with cardiomyopathies.¹⁴² Indeed, a global surveillance of viral diseases by the World Health Organization, identified coxsackievirus B (CVB) as the leading infectious cause of clinical cardiovascular disease.¹⁴³ *In vivo* studies analyzing CVB3 are nearly exclusively based on mice,¹⁴⁴ which offer several advantages: first, many inbred and transgenic strains are available; second, they can be used in large numbers; third, murine model systems show both acute myocarditis and long-term disease, thus paralleling the human disease.¹⁴⁵ The outcome of an infection varies with the virus genotype,^{146,147} mouse strain, gender, age, and immune status.^{148–151} One of the cardinal features of CVB3 is infectivity of the CNS in neonatal mice.¹⁵² Important issues such as protection based on DNA vaccines,¹⁵³ the role of B cells during infection,¹⁵⁴ and the role of CD8⁺ T cells in myocarditis¹⁵⁵ have all been analyzed in the mouse. A few studies performed in Asia focusing on abnormal heart function due to myocarditis were performed in golden hamsters.^{156,157} CVB3 can be grown in cell cultures; the virus–receptor interaction and cell damage after infection can be analyzed in a number of cell lines derived from different species including monkeys, hamsters,¹⁵⁸ and humans.¹⁵⁹

Reoviridae, Reoviruses

In analogy to CVB3, *reovirus*-induced murine myocarditis provides an excellent model for the human disease, and depending on the virus strain, dose, route of inoculation, and age of the host, cardiac damage varies,¹⁶⁰ being most dramatic in suckling mice.¹⁶¹ Indeed, De Biasi et al. demonstrated in a very well-characterized experimental model that severe myocardial damage in neonatal mice is induced by apoptosis.¹⁶² Another very important finding based on nude and severe combined immunodeficiency (SCID) mice is that, in contrast to CVB3, lymphocytes seem to be only protective and not responsible for tissue damage.¹⁶³ The virus not only has a ubiquitous natural host range — including monkeys, swine, dogs, cats, rabbits, birds, and even insects — but can also be grown in a wide variety of cell lines,¹⁶⁴ including Madin–Darby canine kidney,¹⁶⁵ rhesus monkey kidney, and human embryonic intestinal cells.¹⁶⁶ Importantly, cytopathogenic effects seen in tissue culture correlate very well with the viral capacity to induce myocarditis *in vivo*,^{167,168} which may allow

scientists to replace some animal experiments with *in vitro* studies. Avian reoviruses can induce myocarditis and pericarditis, too, but only a few experiments to date have been conducted in birds.^{169,170}

ANIMAL MODELS FOR HEMORRHAGIC FEVER VIRUS INFECTIONS

Bunyaviridae

Rift Valley Fever Virus (RVFV)

Rhesus macaques may be used as an animal model for hemorrhagic syndromes related to RVFV infection. Upon intravenous inoculation with RVFV most macaques develop a benign viremic infection, but 20% of infected animals experience disseminated intravascular coagulation and extensive liver necrosis.¹⁷¹ Young gerbils develop encephalitis upon peripheral or intracerebral viral inoculation. Older animals are less susceptible to peripheral infection, but histological evidence of mild necrotizing encephalitis without detectable infectious RVFV in the brain was reported.¹⁷² Laboratory mice can be infected with wild-type RVFV upon subcutaneous or intraperitoneal inoculation, inducing fulminant hepatitis and late-developing encephalitis. Encephalitis occurred more often in mice treated with antiviral drugs.¹⁷³ In laboratory rats, the pathogenesis of RVFV infection was highly variable, with disease manifestations varying from infection without any symptoms to fatal hepatitis or encephalitis.¹⁷⁴ Sheep manifest many of the clinical symptoms associated with hemorrhagic fever virus infections such as watery mucoid discharges, bloody diarrhea, and external hemorrhage.¹⁷⁵

Bunyamwera Virus

Experimental Bunyamwera virus infections were performed in mice. Wild-type mice required intracerebral inoculation to develop fatal infection, while IFN- α/β receptor knock-out mice could also be infected by intraperitoneal inoculation.¹⁷⁶

Crimean–Congo Hemorrhagic Fever (CCHF) Virus

The infectivity of the wild-type CCHF virus has been studied in 11 species of African rodents and in laboratory rabbits, guinea pigs, and hamsters by subcutaneous, intraperitoneal, intravenous, or intracerebral inoculation. No clinical signs of infection were seen, although viremia was occasionally detected. Researchers in the Soviet Union reported using a mouse-adapted strain for the experimental infection of European hares, long-eared hedgehogs, rabbits, and Syrian hamsters. The mouse-adapted strain produced high viremia and allowed demonstration of viral transmission to ticks.^{177,178} The viral transmission cycle from birds to ticks and from ticks to rabbits was further analyzed in experimentally infected African wild birds.¹⁷⁹ Antiviral compounds such as ribavirin have been tested in the mouse model.¹⁸⁰ Infant mice infected intraperitoneally with CCHF virus display viremia and high viral titers in the liver and later lower viral titer in the brain and heart.¹⁸¹

Hantavirus

In several monkeys from the Old and New World, intravenous infection with Prospect Hill hantavirus or Puumala virus was asymptomatic, with the exception of a chimpanzee and several cynomolgus monkeys.¹⁸² Cynomolgus macaques showed clinical signs of hemorrhagic fever with renal syndrome such as lethargy, anorexia, proteinuria, and/or hematuria.^{183–185} Although rodent

models have been extensively used to study the immune response against hantavirus and to evaluate antiviral compounds, they do not mimic the pathogenesis of hantavirus infection in humans.¹⁸⁶ Following exposure to Andes virus, adult Syrian hamsters have been shown to develop rapid respiratory distress with pathologic findings of pulmonary edema and pleural effusion with similarities to hantavirus pulmonary syndrome.^{187,188} Newborn mice and rats inoculated intracerebrally or intraperitoneally with hantavirus displayed virus titers in various organs; the severity of the infection was dependent upon animal strain and age.¹⁸⁹

Sandfly Fever Virus

Humans constitute the only known vertebrate host for sandfly fever virus. Therefore, several experimental infectious studies with sandfly virus have been performed in humans. Although cynomolgus monkeys are also infectable with the Sicilian virus by intramuscular challenge, no clinical signs of disease are seen.^{190,191} No evidence of disease has been obtained with sandfly fever virus by intracerebral, intracutaneous, intratesticular, intranasal, and intraperitoneal inoculation in nonhuman primates such as baboons, vervets, and chimpanzees. In laboratory animals such as hamsters, mice, rats, rabbits, and guinea pigs, poor infectivity for clinical isolates was demonstrated. Only intracerebral inoculation of a mouse-adapted strain (by several passages in suckling mouse brain) was able to produce disease in adult mice.¹⁹² Alternatively, the phlebovirus Punta Toro produces a nonencephalic lethal infection in mice with fulminant hepatocellular necrosis, reproducing several aspects of human sandfly fever infection. This animal model was established for preclinical analysis of antiviral compounds in sandfly fever infections.¹⁹³

Arenaviridae

Guanarito Virus and Machupovirus

With minor variation, Guanarito virus infection is similar to that of other arenaviruses. Pichinde, Junin, Machupo, and Lassa viruses show a similar course in the guinea pig and primate models, characterized by lymphoid necrosis, bone marrow depletion, and interstitial pneumonia, with macrophages as the predominant cell involved in all three diseases.¹⁹⁴ Rhesus monkeys inoculated with Guanarito virus became viremic, developed clinical signs such as lethargy, and had fever but failed to produce hemorrhagic disease and survived the infection.¹⁹⁵ Guinea pigs infected by subcutaneous application with Guanarito virus developed histological lesions of single cell necrosis in the gastrointestinal tract, interstitial pneumonia, lymphoid and hemapoietic cell necrosis, and occasional hemorrhage with virus present in multiple organs.¹⁹⁶ Newborn or adult cane mice, a natural host of Guanarito virus, developed nonlethal chronic viremic infection.¹⁹⁷ Machupo virus has been shown to produce neurological signs and death in infant hamsters and mice following intracerebral infection.¹⁹⁸

Lassa Fever Virus

Similarities to human disease were observed in baboons and squirrel monkeys infected with Lassa fever virus. Symptoms in baboons included fever, general toxicity, and hemorrhagic diathesis in the presence of high-level viremia.^{199,200} Laboratory mice and guinea pigs were infected using native or mouse-adapted viral strains.²⁰¹ The guinea pig model was applied to investigate the protective potential of Lassa-immune plasma of guinea pig, primate, and human origin.²⁰² An alternative animal model is based on the nonhuman pathogenic arenavirus Pichinde, which acquires virulence for guinea pigs or hamsters upon sequential spleen passages in those species and produces a Lassa fever–like disease, including terminal vascular leak syndrome in certain inbred strains of hamsters and guinea pigs.^{203,204}

Junin Virus

Macaques infected by aerosol with Junin virus developed severe clinical disease, whereby the virus was detected in visceral organs of early-dying animals and in the central nervous system of animals dying at a later time point. Infected rhesus macaques mimicked closely the clinical syndromes observed in humans and have been used to investigate the protective capacity of vaccines.^{205,206} In guinea pigs, different Junin strains were shown to reproduce clinical syndromes of hemorrhagic fever, visceral pathology, or neurological illness.²⁰⁷ Newborn and adult mice infected with Junin virus were used to analyze the beneficial effects of antiviral compounds, such as cyclophosphamide, for the treatment of Junin viral infection.²⁰⁸ The neuroinvasiveness of several laboratory and wild-type strains of Junin virus was studied in albino mice, guinea pigs, and the South American wild rodent *Calomys musculus* of different ages.²⁰⁹

Flaviviridae

Yellow Fever and Dengue Fever Viruses

Yellow fever pathology and treatments can be modeled in macaques, which reproduce a fulminant form of the disease. Hamsters are infectable by intraperitoneal inoculation and display viremia and coagulation abnormalities; however, in contrast to macaques, many adult hamsters survive the infection and represent therefore a valuable alternative to the macaque model.²¹⁰ Mice have mainly been used as an *in vivo* model for vaccine studies. Nonadapted yellow fever viral strains cause fatal encephalitis in naïve mice, while immunized mice may develop a nonfatal encephalitis.²¹¹

Although several species of nonhuman primates have been experimentally infected with dengue fever virus and have developed a high-titer viremia, only humans show clinical illness upon peripheral infection. After intracerebral challenge, small animals such as mice are often used as models to study dengue virus infection. In baby mice, unadapted strains usually produce subclinical infection, whereas mouse brain-adapted strains may lead to paralysis and death.^{212,213}

The Montana Myotis leukoencephalitis virus (MLLV), a bat flavivirus, has been proposed as alternative model for yellow fever virus and dengue fever virus infection in SCID mice. MMLV appears to be equally sensitive to antiviral agents and may therefore be useful for preclinical analysis of antiviral compounds.²¹⁴

Marburg Virus

African green monkeys, rhesus monkeys, and cynomolgus species are susceptible to Marburg virus infection. The monkeys reproduce many clinical signs observed in humans, including weight loss, fever, hemorrhages, and skin rash, hypothermia, shock, and death.^{215,216} In baboons the course of infection tends to be less severe.²¹⁷ Guinea pigs develop a mild febrile illness when infected with wild-type Marburg virus, while animal-adapted viruses were shown to produce major pathologic features of lethal infection resembling these in mice and primates.²¹⁸ Guinea pigs and hamsters were mainly used to propagate the virus, titrate stock inoculate, or produce specific antibody.²¹⁹

Ebola Virus

Ebola Reston, Zaire, and Sudan efficiently infect nonhuman primates; animals infected with Zaire virus demonstrate the highest mortality.²²⁰ In macaques, the disease course of Ebola was very similar to that observed in humans; cynomolgus monkeys also reproduce typical symptoms such as anorexia, splenomegaly, petechial facial hemorrhages, and severe subcutaneous hemorrhages.²²¹ Several vaccine formulations were tested in cynomolgus monkeys. Early pathogenic events of Ebola infection were investigated in guinea pigs; clinical isolates produced nonlethal febrile illness, while

guinea pig–adapted strains were associated with progressive disease and death.²²² Newborn mice are susceptible to Ebola Zaire infection. Adult mice, which are used to determine the immune protection of vaccine candidates, require mouse-adapted strains for infection.²²³

ANIMAL MODELS FOR RESPIRATORY VIRAL INFECTIONS

Respiratory Syncytial Virus (RSV)

The natural host range of respiratory syncytial virus (RSV) includes at least the higher primates and bovine species, in addition to humans. Among the apes, chimpanzees have been used most extensively, as they are permissive for RSV, develop upper and lower respiratory tract infections, and have a core temperature similar to that of humans. Other primates used include the semipermissive African greens, rhesus, cynomolgus, or bonnet monkeys and New World monkeys such as marmosets, tamarins, and owl monkeys, which might be less than ideal because their body temperature is higher than in humans.²²⁴ The infant ferret and the cotton rat provide together the basic tools to investigate the pathogenesis of RSV disease. After intranasal application, ferrets yielded virus from their tracheas, nasal turbinates, and lungs, with histopathologic changes in the nasal passages. The infant ferret model lacks bronchiolar involvement; however, it mimics the lower respiratory tract infection with RSV seen in infants and the upper respiratory tract disease in older people. In contrast, RSV replicated to a high titer throughout the respiratory tracts of cotton rats. Pathologic changes of rhinitis, bronchitis, and bronchiolitis were present, which predominantly involved the surface epithelium.²²⁵ Rodent models have been used to study quantitative virology, immunology, and airway pathophysiology, with weight loss as a surrogate marker for RSV disease.²²⁶ Guinea pigs infected by instilling human RSV onto the nasal mucosa showed airway hyperresponsiveness that correlated with histological changes of acute bronchiolitis, but they generally do not have severe clinical symptomatology, such as respiratory distress.²²⁷ Mice also develop pulmonary pathology upon nasal challenge; they have the advantages of a body temperature similar to that of humans and the ready availability of immunological reagents.²²⁸ The lamb, calf, and sheep are potential models to study the impact of viral infection on lung mechanics, as these animals can be affected by RSV and can undergo evaluation of lung function. Inoculation of sheep with bovine or ovine RSV causes histological changes in the lung, ranging from bronchiolitis to interstitial pneumonitis.²²⁹ Infection of calves via aerosol rather than intranasal or intratracheal application of fluid medium induced lower respiratory tract infection, with lobular distributed atelectasis.²³⁰

Influenza Virus

The ferret has been a preferred animal model for influenza virus infections because, upon nasal application, ferrets exhibit typical clinical symptoms, such as nasal discharge, loss of appetite, congested eyes, otological manifestations, and importantly, fever.²³¹ In squirrels, mild infections with human influenza strains were reproduced, while cynomolgus macaques were also infected with the highly pathogenic influenza A (H5N1) and developed necrotizing bronchointerstitial pneumonia and lesions in extraperitoneal organs upon viral application onto tonsils and conjunctiva.²³² Mice are often used to study the immunological response to vaccine formulations or to perform transmission experiments. Clinical isolates that are not mouse adapted were shown to induce toxic pneumonitis in absence of significant viral replication and high variability of virus titers in infected tissues.²³³ In contrast, strains adapted by serial passages in mice multiply to high titers, leading to lethal infection of the lower respiratory tract. As the inoculum is usually instilled directly into the lung, it is difficult to use this model to evaluate the role of mucosal immunity.²³⁴ Chicken and pigs are valuable animal models, particularly when an avian or swine influenza virus is to be studied.²³⁵

Parainfluenza Virus

Parainfluenza virus (PIV) 1, strain Sendai, infects mice naturally or experimentally, and both airborne transmission and transmission by contact occur rapidly. The virus replicates in the lungs of mice with extensive damage to the mucus membrane, but without obvious symptoms. In contrast, rats infected intranasally with PIV 1 develop lung lesions and clinical illness. PIV 2, 3, and 4 naturally infect humans, rabbits, guinea pigs, hamsters, and monkeys with few symptoms.²³⁶ Neonatal ferrets have been shown to develop progressive illness and death when infected with wild-type PIV 3, but infection with virus passaged in embryonic eggs was silent but persisted for several days, indicating viral replication.²³⁷ Intranasal inoculation of young hamsters and guinea pigs with PIV 3 resulted in infection with little sign of illness. Microscopic examination revealed focal necrotic lesions of the epithelium in the anterior turbinates and major bronchioles with peribronchial and perivascular leukocyte infiltration.²³⁸ Guinea pigs have provided an animal model to study the mechanisms of virus-induced asthma and airway hyperreactivity and together with cotton rats were used to study effects of various therapeutic regimens.²³⁹

Adenoviruses

These DNA-containing viruses constitute a large family of specific serotypes whose natural hosts include man, monkeys, cattle, rodents, dogs, and chickens. Generally, species specificity exists between hosts and given serotypes, such that experimental animal models are not readily available. Chimpanzees and gibbons can be infected with some of the human serotypes.²⁴⁰ Cotton rats were infected with several adenovirus serotypes, but only serotype 5 produced pulmonary histopathology similar to reported findings in human infections.²⁴¹ In mice, type 5 adenovirus was unable to replicate but nevertheless produced pneumonia.²⁴² Pulmonary lesions were also induced in pathogen-free, colostrum-deprived piglets by serotypes 1, 2, 5, and 6.²⁴³

Coronaviruses

Coronaviruses are common respiratory and enteric pathogens of a variety of birds and mammals, including humans. In general, each coronavirus causes disease of only one animal species. Several coronaviruses cause fatal systemic disease in animals, including feline infectious peritonitis virus (FIPV), hemagglutinating encephalomyelitis virus (HEV) in swine, avian infectious bronchitis virus (IBV), and mouse hepatitis virus (MHV).²⁴⁴ Challenge immunity experiments were performed in mice using a MHV strain, which displays tropism for the upper respiratory tract following intranasal inoculation.²⁴⁵ Mucosal immunity was also analyzed in the swine model using porcine respiratory coronavirus (PRCV). Upon natural or experimental infection (oral–nasal inoculation or aerosol), suckling pigs in most cases only develop mild or no respiratory disease, although pneumonia and mortality were also reported.²⁴⁶

Rhinoviruses

The host range of human rhinoviruses appears to be rather limited. Higher primates such as chimpanzees and gibbons were infected with some of the human serotypes, but experimental infection of a variety of laboratory animals has been unsuccessful.²⁴⁷ The vervet monkey was infected with the equine rhinovirus but not with the human rhinovirus by spraying viral suspension onto the throat and nose. The equine rhinovirus has been shown to infect a wide range of animals but differs from the human virus in several aspects, such as the lack of preference for growth at low temperatures.²⁴⁸ In mice, replication of rhinovirus type 2 variants, adapted to the animal by

serial passages in tissue cultures, was described, and replication could be further increased by treating mice with actinomycin D.²⁴⁹ Several experimental upper respiratory infections have been performed in human volunteers.²⁵⁰

Coxsackievirus and Echovirus

Due to the lack of appropriate animal models, human volunteers have been used for experimental coxsackie A-2 virus or echovirus infection. Coxsackie A-2 virus caused weak illness in volunteers after intranasal or aerosol administration, with symptoms such as sore throat, malaise, headache, and fever. In humans, nasal echovirus infection was characterized by nasal discharge, sneezing, and coughing over a period of one week. The virus could be recovered from 80% of infected volunteers, but fever was generally not present. Although mice may be infected with coxsackievirus by intracerebral inoculation, pathogenicity appears to be rather limited.²⁵¹

Measles Virus

Many nonhuman primates are susceptible to measles virus. Macaques infected by conjunctival or intranasal inoculation reproduce the common form of measles pneumonia seen in infants. Leukopenia was observed in a minority of animals, but widespread dissemination to epithelial cells, endothelial cells, and lymphoid tissue was documented. Rhesus and cynomolgus monkeys have served as animal models for studies of the pathogenesis of measles, immune suppression, analysis of viral virulence, and vaccine-induced immune protection.²⁵² In rodents, such as hamsters, mice, and rats, encephalitis can be induced upon intracerebral infection with rodent-adapted neurotropic strains, but with the exception of cotton rats, it is not possible to obtain infection of the respiratory system.²⁵³ Since cotton rats are fully immunocompetent and MV replicates in their respiratory tracts, this model was used to investigate the immunosuppressive effect of MV on B and T cells *in vivo* and for initial preclinical testing of candidate measles vaccines.²⁵⁴

Herpes Simplex Virus (HSV)

The newborn guinea pig should provide a useful model to explore the pathophysiology of HSV infection. Intranasal inoculation of newborn guinea pigs with HSV resulted in infection of the upper respiratory tract, involvement of the central nervous system, and disseminated infections with high mortality.²⁵⁵ Young mice infected by various routes with HSV have been useful for screening potential therapeutic agents. Newborn mice inoculated intranasally with HSV show replication of the virus in the lung, leading to pneumonia and pulmonary influx of lymphocytes with early bloodborne dissemination to the liver, spleen, and brain.²⁵⁶ Important limitations of the mouse model include the high mortality and the fact that the immune response to HSV in mice and humans may be quantitatively and qualitatively quite different.²⁵⁷

Cytomegalovirus (CMV)

Cytomegalovirus (CMV) is one of the major viral pathogens that can infect immunosuppressed patients, who develop disseminated infections, with virus-induced lesions in almost every organ system.²⁵⁸ Rabbits as well as rats, mice, and guinea pigs have been infected by inoculation with nonhuman herpes viruses, leading to disseminated disease affecting many tissues, including the liver, spleen, and lung. Typically, disease in these animal models does not involve respiratory infection and shows high death rates. In irradiated rats, infection with rat CMV resulted in disseminated infection and interstitial pneumonia, causing death in nearly 90% of the animals.²⁵⁹

Epstein–Barr Virus

Epstein–Barr virus (EBV) infections of New World primates, such as cottontop tamarins or the common marmoset, have been used to develop potential vaccine strategies. In this model, the natural route of transmission is bypassed by parenteral virus injection. Old World nonhuman primates proved to be refractory to infection with EBV due to cross-reacting antibodies. Infection of nonhuman primates with lymphocryptoviruses (LCVs) closely related to EBV has been established for study of EBV pathogenesis. Rhesus LCV-naïve animals can be orally infected, thus reproducing the natural mode of transmission and providing a model system for studying the earliest phases of acute infection, the establishment of persistent infection, and the development of host immune response.²⁶⁰ Mice can be infected orally with murine herpesvirus 68 (MHV-68). In analogy to the human infection, MHV-68 infects upon intranasal application with the respiratory tract as its primary target and disseminates to the lymphoid compartment.²⁶¹ Rabbits orally infected with EBV or EBV-related herpes virus from cynomolgus monkeys showed continuous infection in peripheral blood and development of lymphomas.²⁶²

MUCOCUTANEOUS VIRAL INFECTIONS

Papillomaviruses

The main problem with animal models for papillomaviruses is their strict species specificity. Even under experimental conditions, these viruses do not infect any host other than their natural one. Because of that, no animal model of human papillomavirus (HPV) exists, and research has to rely on animal papillomavirus systems.²⁶³ The most studied models are bovine papillomavirus (BPV), cottontail rabbit papillomavirus (CRPV), and canine oral papillomavirus (COPV). Animal papillomaviruses provide excellent *in vivo* models for HPV infection, carcinogenesis, and vaccination. The animal systems have shown that vaccination against papillomavirus is feasible and effective for both prophylactic and therapeutic vaccines.^{264,265} One of the main focuses with animal models of papillomavirus infection lies in the role of the virus in carcinogenesis. This aspect will be discussed in more detail in the section on oncogenic viruses.

Herpes Simplex Virus

Although herpes simplex virus infects different organs, the focus of this paragraph is on animal models for the cutaneous localization of the virus. A variety of animal models have been used to evaluate the efficacy of antiviral chemotherapy for the treatment of mucocutaneous herpes simplex virus infection. These models include the infection of the dorsal cutaneous surface of the guinea pig,^{266,267} the lumbosacral skin of the hairless mouse,^{268,269} the lumbosacral skin of the athymic nude mouse,²⁷⁰ the orofacial skin of CD-1 and hairless mice,^{268,271} and the ears of Swiss white mice.²⁷² Another animal model that shares many similarities with human genital herpes infection, including a natural route of inoculation (intravaginal) and self-limiting genital skin disease (vulvovaginitis) that is associated with neurologic and urologic complications, is the guinea pig vaginal model.²⁷³ This model is also suitable for the evaluation of herpes simplex virus vaccines.²⁷⁴ A less expensive animal model for mucocutaneous infection with herpes simplex virus is mice, where female mice are intravaginally infected with HSV-1 or HSV-2.^{275,276} This system has been widely used to study the efficiency of vaccination protocols against HSV infection and to examine the pathohistology of HSV infection.²⁷⁷

Rotavirus

Candidate vaccines for rotavirus can be studied in mouse and rabbit models of enteric viral infections.^{278,279} Those models, however, often do not predict vaccine efficacy against disease evaluated in neonatal large animals or children.²⁸⁰ The outcome of vaccine efficacy studies using the adult mouse model also strongly depends on the genetic background of the mouse strain.²⁸¹ An alternative model that lacks the disadvantages of the mouse model is the gnotobiotic pig model.²⁸⁰

ONCOGENIC VIRAL INFECTIONS

Most of the oncogenic virus infections have been discussed in the previous paragraphs, where animal models for viral infections of different organ systems are described.

Generally, to study oncogenicity of viruses in animal models two different approaches are possible:

1. Infection of animals with the oncogenic virus itself
2. Use of animals transgenic for known or supposed viral oncogenes

Papillomaviruses

Because of the strict species specificity of papillomaviruses, research has to rely on animal papilloma virus systems. In cattle feeding on bracken fern, bovine papillomavirus (BPV)-4 causes cancer of the upper gastrointestinal tract.²⁸² In animals with the same diet, infection with BPV-1 and BPV-2 leads to cancer of the urinary bladder.²⁸³ Bracken fern is known to contain immuno-suppressants and chemical carcinogens, which facilitate virus-induced carcinogenesis.²⁶³

Infection of rabbits with cottontail rabbit papillomavirus (CRPV) leads to skin carcinogenesis,²⁸⁴ whereas infection of dogs with canine oral papillomavirus (COPV) induces papillomas and squamous cell carcinomas.²⁸⁵

Hepatitis B Virus

The hepatitis B virus (HBV) belongs to the family *Hepadnaviridae*. Besides HBV, there exist several related viruses that infect animals and can be used to study hepadnavirus-associated hepatocellular carcinoma (HCC). The first model described was the natural woodchuck hepatitis virus (WHV) infection. In several zoo animals, hepatocellular neoplasms have been described.²⁸⁶ Other naturally occurring hepadnavirus infections leading to HCC have been described in California ground squirrels,²⁸⁷ the Richardson ground squirrel,²⁸⁸ and the arctic ground squirrel.²⁸⁹ To exploit the woodchuck as an experimental model, the animals had to be bred in laboratory animal facilities. A breeding colony of WHV-negative woodchucks was established at Cornell University.²⁹⁰ In this model experimentally WHV-infected animals had a lifetime risk of HCC of 100%.

In a small percentage of tree shrews, a primitive primate, infection with HBV together with the administration of aflatoxin induced HCC.²⁹² Although chimpanzees are a suitable but expensive model to study HBV infection, few descriptions of HCC in chimpanzees have been reported in the literature.²⁹⁰

Recently, a number of investigators developed strains of inbred transgenic mice that express the individual HB envelope,^{293,294} core,²⁹⁵ precore,²⁹⁶ or X proteins^{297,298} or the entire HBV genome.^{299–301} These transgenic mice have been widely used to study the development and the mechanisms of HBV-induced HCC.

Hepatitis C Virus

Unlike HBV, there have been no reports of closely related HCV-like viruses that naturally infect animals. This restricts the available model systems that could be used to understand development of HCV-induced HCC to transgenic mouse models. In fact, there are only few reports of a transgenic mouse model where the animals develop HCC; the occurrence of poorly differentiated HCC within adenomatous nodules has been reported.³⁰² In another, more artificial model, HCC in transgenic mice could be induced by repeated hepatocyte injury.³⁰³

Epstein–Barr Virus

Several models exist to study EBV-associated B-cell lymphoproliferative disease. A small-animal model is based on SCID mice inoculated with PBMC from healthy EBV-seropositive blood donors.³⁰⁴ This leads to EBV+ B cell lymphomas in 45% of the mice.³⁰⁵

Murine herpesvirus 68 (MHV-68) is a virus that resembles EBV in many ways and therefore is a useful model for the study of gammaherpesvirus *in vivo*. About 9% of persistently infected mice developed lymphoproliferative disease, and this was increased to 60% with CyA treatment.³⁰⁶

Some New World nonhuman primates can be infected with EBV. In cotton top tamarins, EBV infection could lead to malignant lymphoma,³⁰⁷ whereas infection of the common marmoset did not induce lymphoma. However, New World primates are endangered species and are rare and expensive. Recently, rhesus monkeys have provided a new model for EBV infection using rhesus lymphocryptovirus for oral inoculation. However, without overt immunosuppression, lymphocryptovirus-related malignancies have not developed in this model.³⁰⁸

Studies of tumorigenic mechanisms of EBV have also been promoted by the use of transgenic mice.^{309,310}

LYMPHOTROPIC VIRAL INFECTIONS (EXCLUDING AIDS)

Lymphotropic Herpesviruses

The γ -herpesvirus family includes a series of lymphotropic viruses, among them the murine gammaherpesvirus 68 (γ HV68), which establishes latent infection of the B cells in mice. Similar to EBV infection in humans, the virus causes polyclonal B-cell expansion and is under control of both CD4 and CD8 T cells.³¹¹ Herpesvirus sylvilagus is another lymphotropic virus that infects both B and T lymphocytes in cottontail rabbits (*Sylvilagus floridanus*). It induces a lymphoproliferative disorder similar to acute mononucleosis.³¹² Herpesvirus saimiri is another member of the γ -herpesvirus family; it infects various primate hosts. Although the natural host, the squirrel monkey (*Saimiri sciureus*), can be persistently infected with the virus without disease, the same virus can cause acute fatal T-cell lymphomas in other monkey species.³¹³ Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8) cause Kaposi's sarcoma in humans and a variety of lymphoproliferative disorders in humans coinfecting with HIV. An animal model using C.B-17 SCID/SCID mice implanted with human fetal thymus and liver grafts has been described, where infection of human CD19-positive B cells has been documented.³¹⁴

Lymphotropic Retroviruses

Human T-cell leukemia virus type I (HTLV-1), which can cause various manifestations in humans including adult T-cell lymphoma and leukemia, has been successfully studied in C3H/HeJ mice, which were inoculated to become carriers. This model will be useful to study the latent stage of this disease.³¹⁵ Caprine arthritis–encephalitis virus (CAEV) is another retrovirus belonging to

the lentiviruses that causes chronic inflammatory changes in various organs including the joints and the brain after neonatal infection of goats. This model is useful for the study of inflammatory polyarthritis and lentiviral pathobiology.³¹⁶

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Transgenic Models of Prion Diseases

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INTRODUCTION

Prion diseases have been studied for many years because of their extraordinary biology and the unique properties of the infectious agent. Because prions can only be assayed in experimental animals and the time between inoculation and disease is long, characterization of the infectious agent has been enormously difficult. On the other hand, dramatic advances in our understanding of prions and the prion diseases have occurred, in large part, precisely because they can be transmitted to experimental animals. The advent of transgenic technology has done much to further our understanding about various aspects of prion biology. Manipulation of prion protein (PrP) genes by transgenesis in mice has been accomplished using two approaches: PrP gene knockout or gene replacement using homologous recombination in embryonic stem cells and microinjection of transgenes into fertilized embryos. These studies have provided important insights into the pathogenesis of prion diseases, including the mechanism of prion propagation, the molecular basis of the species barrier, prion strains, and the mechanism by which human prion diseases can be both genetic and infectious. In this review we will focus on the role that various transgenic approaches have played in furthering our understanding of prions and prion diseases and will also outline the remaining unresolved issues in prion biology.

PRIONS AND THE PRION DISEASES

The prion disorders of animals and humans (Table 6.1) share a number of common features. They all have long incubation periods ranging from months to years and are invariably fatal once clinical symptoms have appeared. Because they are transmissible by inoculation, prion diseases are also known as “transmissible spongiform encephalopathies” (TSEs). The neuropathological changes that accompany disease in the central nervous system (CNS) are remarkably consistent

Table 6.1A Animal Prion Diseases

Disease	Host	Etiology
Scrapie	Sheep and goats	Thought to involve horizontal and vertical transmission
Bovine spongiform encephalopathy (BSE)	Cattle, kudu, gemsbok, nyala, oryx, eland, ankole cow, bison	Feed-borne in contaminated meat and bone meal; also maternal transmission
Feline spongiform encephalopathy (FSE)	Domestic cats; exotic cats: cheetah, puma, tiger, ocelot, African lion	BSE-contaminated feed
Transmissible mink encephalopathy (TME)	Mink	Prion-contaminated feed
Chronic wasting disease (CWD)	Captive and free-ranging mule deer, white-tailed deer, and elk	Thought to involve horizontal transmission

Table 6.1B Human Prion Diseases

Disease	Etiology	Age of Onset/Incubation Period and Duration of Illness	Clinical Features	Neuropathology
Sporadic CJD	Hypotheses include somatic mutation or spontaneous conversion of PrP ^C to PrP ^{Sc}	Age of onset 30–80 years; median age of onset 60–65	Intellectual deterioration; myoclonus; variable cerebellar, oculomotor dysfunction; 70% die within 6 months	Amyloid plaques in 10% of cases; characteristic EEG pattern
Familial CJD	Autosomal dominant PRNP mutations	Earlier onset, longer duration		
GSS	Autosomal dominant PRNP mutations	Earlier onset, longer duration	Cerebellar dysfunction; pyramidal motor dysfunction; intellectual deterioration	Amyloid plaques
FFI	Autosomal dominant PRNP mutations	Earlier onset, longer duration	Insomnia; autonomic dysfunction; motor dysfunction	Thalamic involvement
Kuru	Infection through ritualistic cannibalism	Incubation periods from 30 to 360 months; duration between 3 and 12 months	Cerebellar ataxia; pyramidal motor dysfunction; intellectual deterioration; death in < 12 months	Amyloid plaques
VCJD amyloid plaques	Infection by BSE prions	Mean age of onset 26 years; mean duration 14 months	Behavioral and psychiatric disturbances; cerebellar ataxia; peripheral sensory neuropathy	“Florid”

among these different diseases. Microscopic examination of the CNS typically reveals neuronal vacuolation and degeneration and a reactive proliferation of astroglia. The lack of a lymphatic inflammatory response is also an important characteristic. Some types of prion disease are also characterized by the deposition of amyloid plaques composed of insoluble aggregates of PrP. Amyloid plaques are a notable feature of the human prion diseases kuru and Gerstmann–Straussler–Scheinker (GSS) syndrome but are infrequently found in the brains of patients with sporadic Creutzfeldt–Jakob disease (CJD). Amyloid plaques are a constant feature of the variant form of CJD (vCJD) in teenagers and young adults in the United Kingdom, but they differ in morphology from kuru and GSS plaques in that the immediately surrounding tissue shows prominent microvacuolation giving the plaques a so-called “florid” appearance.¹

The unifying hallmark of prion diseases is the aberrant metabolism of the prion protein (PrP), which exists in at least two conformational states with different physicochemical properties. The normal form of the protein, referred to as PrP^C, is a sialoglycoprotein of molecular weight 33 to 35 kDa, which is attached to the surfaces of neurons and other cell types by means of a glycosphosphatidyl inositol (GPI) anchor. PrP^C is sensitive to protease treatment and is soluble in detergents. The disease-associated isoform, referred to as PrP^{Sc}, is found only in infected brains; it is partially resistant to protease treatment, is insoluble in detergents, and tends to aggregate.

While the exact nature of the infectious agent remains to be determined, considerable evidence argues that prions lack nucleic acid and are composed largely, if not exclusively, of PrP^{Sc} molecules. In its simplest form, the so-called “protein-only” hypothesis contends that prion replication involves conversion of benign host-encoded PrP^C into pathogenic PrP^{Sc}; the central event is the coercion of PrP^C by PrP^{Sc} to adopt the infectivity-associated conformation. There is debate over the detailed features of the pathway to PrP^{Sc} formation (reviewed in Reference 2). In one model, PrP^C exists in equilibrium with a form of PrP, referred to as PrP^{*},³ that is bound to an ancillary factor, referred to as “protein X.”⁴ The PrP^{*}–protein X complex interacts with PrP^{Sc}, which induces a conformational change in PrP^{*}; the end result is two molecules of PrP with the infectious PrP^{Sc} conformation, which are free to induce conformational changes in additional PrP^{*} molecules during the infectious cycle. The alternative model of nucleation-dependent polymerization argues that PrP^{Sc} conversion requires the presence of a seed of aggregated PrP^{Sc}.

MICROINJECTION TRANSGENIC APPROACHES

The majority of transgenic studies of prion diseases have involved the incorporation of wild-type or mutant PrP genes from different species into the genome of fertilized mouse embryos by DNA microinjection. The seminal transgenic experiments utilized cosmid clones containing PrP gene sequences isolated from Syrian hamster (SHa) and the I/InJ strain of mice,^{5,6} and this approach was also used to produce transgenic mice expressing sheep PrP.⁷ The cos.SHaTet vector is a modification of the SHa cosmid vector and contains a 43 kb DNA fragment encompassing the PrP gene and approximately 24 and 6 kb of 5' and 3' flanking sequences, respectively.⁸ The vector is designed to allow the convenient insertion of PrP coding sequences. A plasmid expression vector based upon the PrP gene derived from the I/InJ PrP cosmid, referred to as phgPrP or the “half-genomic” construct, has also been used to produce transgenic mice.⁹ A modification of the “half-genomic” construct, referred to as MoPrP.Xho, that allows for convenient insertion of cDNA coding sequences has been extensively used to overexpress a variety of genes in the CNS including amyloid precursor protein (APP) and human presenilin 1.¹⁰ Smaller, plasmid-based expression vectors based on the mouse PrP gene (*Prnp*)¹¹ and human PrP gene (*PRNP*)¹² control elements have also been engineered. SHa and mouse PrP minigene constructs in which all intron sequences are removed fail to express PrP in the CNS, demonstrating the requirement for at least the smaller intron for efficient expression.^{5,9}

Structure–Function Studies of PrP

The finding that the introduction of PrP transgenes into Zurich I *Prnp*^{0/0} mice restores susceptibility to scrapie opened the possibility for assessing whether a modified PrP^C molecule remains functional, at least insofar as it continues to be eligible for supporting prion propagation. Experiments in cell culture showed that deletion of the PrP sequence encoding residues that are removed from the amino terminus of PrP^{Sc} by limited proteolysis did not prevent the acquisition of protease resistance and PrP^{Sc} formation.¹³ To further investigate the role of this region, a series of transgenic mice expressing amino terminal deletions of varying extent were produced. Transgenic mice expressing MoPrP deleted between residues 23 and 89, referred to as Tg(MoPrP,Δ 23-88)Prnp^{0/0} mice, propagated prions less efficiently than Tg(MoPrP)Prnp^{0/0} mice did. Scrapie incubation times in Tg(MoPrP,Δ23-88)Prnp^{0/0} mice inoculated with either full-length or truncated murine prions were ~3 times longer than in Tg(MoPrP)Prnp^{0/0} mice.¹⁴ Similarly, transgenic mice expressing MoPrP deleted between residues 32 and 93 had prolonged scrapie incubation times and prion titers ~30-fold lower than in wild-type mice,¹⁵ while Tg(MoPrP,Δ32-80)Prnp^{0/0} mice, which retain one octarepeat, propagated prions efficiently, with scrapie incubation times similar to those of mice expressing full length MoPrP,⁹ suggesting that efficient prion propagation requires that PrP^C contain at least one amino-terminal octarepeat sequence.

Although deletions between codons 69 and 84, 32 and 80, 32 and 93, or 32 and 106 of the PrP coding sequence were able to restore susceptibility to scrapie in *Prnp*^{0/0} mice,^{9,16} deletions between codons 32 and 121 or 32 and 134 caused ataxia and degeneration of the granular layer of the cerebellum within 2 to 3 months after birth.¹⁷ This defect was overcome by the coexpression of wild-type MoPrP, suggesting that truncated PrP might compete with a functionally similar non-PrP molecule for a common ligand. Interestingly, the structure of these mutant *N*-terminally truncated proteins is reminiscent of the PrP-related protein, Doppel. Targeting *N*-terminally truncated PrP to Purkinje cells also leads to PrP-reversible Purkinje cell loss and ataxia, further substantiating the notion that Doppel and truncated PrP cause Purkinje cell degeneration by the same mechanism.¹⁸

A series of PrP coding sequence deletions based on putative regions of secondary structure suggested by modeling studies of PrP prior to the determination of the nuclear magnetic resonance (NMR)-derived structure of recombinant PrP were also expressed in *Prnp*^{0/0} mice.¹⁹ These deletions were engineered in a modified PrP construct that lacks amino acid residues 23 to 88, the residues that are removed from the amino terminus of PrP^{Sc} by limited proteolysis. While transgenic mice expressing deletions between codons 95 and 107, 108 and 121, and 141 and 176 remained healthy, transgenic mice expressing deletions at the carboxyl terminus between codons 177 and 190 and 201 and 217 exhibited neuronal cytoplasmic inclusions of PrP and spontaneously developed a fatal CNS illness similar to neuronal storage diseases. In a separate study, the ability of two such deletion constructs to support prion replication was assessed in transgenic mice.²⁰ Surprisingly, transgenic mice in which residues 23 to 88 were deleted remained resistant to infection, and this block to prion propagation was alleviated by further deleting residues 141 to 176. In both cases, the block to prion propagation was overcome by coexpression of wild-type MoPrP. In contrast to these findings, introduction of PrP devoid of amino acids 32 to 93 to *Prnp*^{0/0} mice restored susceptibility to mouse-adapted prions,¹⁵ albeit with protracted incubation times and 30-fold less accumulation of PrP^{Sc} compared to wild-type mice. The discrepancy between these two models presumably results from differences in transgene design that preserve residues 23 to 31 within the latter deletion mutant.

Transgenic mice expressing mutant PrP with deletions between 23 and 88 and 141 and 176 express a PrP molecule referred to as PrP106, which is comprised of 106 amino acids and is the smallest deletion mutant able to propagate prion infectivity.²⁰ Biophysical analysis of synthetic PrP106 revealed that the molecule has the propensity to spontaneously fold into a β -pleated sheet that oligomerizes into soluble aggregates.^{21,22} Transgenic mice expressing PrP106 demonstrate an artificial transmission barrier, such that they become ill with an incubation time of 66 days following

inoculation with RML PrP106 prions compared to 300 days following inoculation with full-length MoPrP RML prions. Similarly, transmission of wild-type Mo RML prions was more efficient than that of PrP106 miniprions in wild-type mice.²⁰ Transgenic mice expressing a molecule called PrP61, in which sequences between 23 and 88 and 141 and 221 were deleted, spontaneously developed ataxia and exhibited neuronal apoptosis and reactive gliosis, in addition to PK-resistant PrP61 aggregates. PrP61 was not found to be transmissible.²³

Transgenic Models of Inherited Prion Diseases

Approximately 10 to 20% of human prion disease is inherited, with an autosomal dominant mode of inheritance. Inherited human prion diseases are associated with missense and insertion mutations in the coding sequence of *PRNP*. Five of these mutations are genetically linked to loci controlling familial CJD, GSS syndrome, and fatal familial insomnia (FFI), which are inherited human prion diseases that can be transmitted to experimental animals.

Although spontaneous neuromyopathy has been reported in aged mice expressing high levels of mouse PrP-B, ShaPrP, and sheep PrP,^{7,9} this phenotype has not been observed in mice that overexpress other wild-type PrP transgenes. Transgenic mice that express a proline-to-leucine mutation at codon 101 of mouse PrP, equivalent to the human GSS P102L mutation, referred to as Tg(MoPrP-P101L), spontaneously developed clinical and neuropathological symptoms similar to those of mouse scrapie between 150 and 300 days of age.^{24,25} After crossing the mutant transgene onto the *Prnp*^{0/0} background, the resulting Tg(MoPrP-P101L) *Prnp*^{0/0} mice displayed a highly synchronous onset of illness at ~145 days of age which shortened to ~85 days upon breeding to homozygosity for the transgene array. Surprisingly, sick Tg(MoPrP-P101L) mice did not harbor PK-resistant PrP^{Sc} in their brains. In addition, Tg(MoPrP-P101L) *Prnp*^{0/0} mice had increased numbers of PrP plaques and more severe spongiform degeneration. In contrast, transgenic mice overexpressing wild-type mouse PrP at equivalent levels did not spontaneously develop neurodegenerative disease, although they had highly reduced mouse scrapie incubation times after inoculation with mouse prions.²⁵

Importantly, the serial propagation of infectivity from the brains of spontaneously sick Tg(MoPrP-P101L) mice to indicator mice expressing low levels of mutant protein, which otherwise do not get sick (Tg196 mice), demonstrated the production of infectious prions in the brains of these spontaneously sick mice.^{25,26} A 55-residue synthetic peptide comprising mouse PrP residues 89 to 103, containing the P101L mutation refolded into a β -sheet conformation, induced prion disease in Tg196 mice after 360 days. Mice displayed GSS-like neuropathological changes in their brains similar to those in mice that developed spontaneous disease. Mice inoculated with peptide in a non- β -sheet conformation remained healthy for more than 600 days.²⁷ In a limited study, prion infectivity from brain extracts of humans expressing the P102L GSS mutation was also propagated in transgenic mice expressing a chimeric mouse-human PrP gene with the P101L mutation.⁴ Other inherited human prion diseases have also been transmitted to transgenic mice expressing human and chimeric mouse-human PrP.^{4,28,29}

In contrast to Tg(MoPrP-P101L) mice, transgenic mice overexpressing a mutant mouse PrP gene with a glutamate-to-lysine mutation at codon 199, equivalent to the codon 200 mutation linked to familial CJD (E200K), did not spontaneously develop neurologic disease.²⁵ A third human prion disease mutation associated with GSS, in which the Tyr residue at codon 145 is mutated to a stop codon, has been modeled in transgenic mice, designated Tg(MoPrP144#). However, no PrP expression was detected in high-copy-number lines, and neither uninoculated Tg(MoPrP144#) mice nor mice inoculated with mouse RML scrapie developed symptoms of neurodegenerative disease.¹⁹ Expression of a mouse PrP version of a nine-octapeptide insertion, designated Tg(PG14) mice, associated with human prion dementia produced a slowly progressive neurological disorder in transgenic mice.³⁰ Upon breeding to homozygosity, Tg(PG14) mice develop ataxia and clinical illness at ~65 days compared to 240 days for hemizygous mice,³¹ and mice accumulate proteinase

K (PK)-resistant and detergent-insoluble PrP, which increases 20 to 80 fold from birth and appears to be associated with apoptotic loss of granule cells in the cerebellum. Partially PK-resistant, detergent insoluble, mutant PrP accumulates in spinal cord, skeletal muscle, and heart, and accumulation is associated with primary skeletal muscle myopathy.³²

Transgenic Studies of Prion Species Barriers

The species barrier describes the ability of prions from one species to cause disease in a different species. In experimental studies, the initial passage of prions between species is inefficient and is associated with prolonged incubation times, with only a few animals developing illness. On subsequent passage of the adapted infectivity in the same species, all the animals became ill after greatly shortened incubation times. Prion species barriers have been abrogated in transgenic mice by expressing PrP genes from other species or artificially engineered chimeric PrP genes. Experiments designed to probe the molecular basis of the species barrier have also provided important clues about the mechanism of prion propagation involving association and conformational conversion of PrP^C into PrP^{Sc}.

As a result of the species barrier, wild-type mice are normally resistant to infection with scrapie prions adapted for transmission in Syrian hamsters (SHa). Seminal experiments by Prusiner and colleagues demonstrated that expression of SHa PrP^C in transgenic mice, referred to as Tg(SHaPrP) mice, rendered them susceptible to SHa prions and produced CNS pathology similar to that found in Syrian hamsters with prion disease.⁵ Expression levels of SHa PrP were inversely correlated with the incubation period of SHa prions.³³ Furthermore, inoculation of Tg(SHaPrP) mice with mouse prions resulted in propagation of prions pathogenic for mice, while inoculation with SHa prions resulted in the propagation of prions pathogenic for hamsters. This work strongly implied that a direct protein/protein interaction between PrP molecules was involved in prion propagation, and that for optimum progression of the disease, the interacting species should be identical in primary structure.³³ Expression of chimeric SHa/mouse PrP transgenes in mice produced prions with new properties. The MH2M transgene carries five amino acid substitutions found in ShaPrP, lying between codons 94 and 188. Tg(MH2M) mice generated prions with an artificial host range such that infectivity produced by inoculation with SHa prions could be passaged from Tg(MH2M) mice to wild-type mice and infectivity produced by inoculation with mouse prions could be passaged from Tg(MH2M) mice to Syrian hamsters.³⁴

The infrequent transmission of human prion disease to rodents is also an example of the species barrier. Based on the results with Tg(SHaPrP) mice, it was expected that the species barrier to human prion propagation would be abrogated in transgenic mice expressing human PrP, referred to as Tg(HuPrP) mice. However, transmission of human prion disease was generally no more efficient in Tg(HuPrP) mice on a wild-type background than in nontransgenic mice. In contrast, propagation of human prions was highly efficient in transgenic mice expressing a chimeric mouse-human PrP gene, referred to as Tg(MHu2M) mice, in which the region of the mouse gene between codons 94 and 188 was replaced with human PrP sequences.³⁵ These mice became ill with an average incubation time of 200 days after inoculation with brain homogenates from patients dying of CJD. These studies made possible the rapid and relatively inexpensive transmission of human prion diseases for the first time. The barrier to CJD transmission in Tg(HuPrP) mice was abolished by expressing HuPrP on a *Prnp*^{0/0} background, demonstrating that mouse PrP^C inhibited the transmission of prions to transgenic mice expressing human PrP^C but not to those expressing chimeric PrP.⁴ To explain these and the results of transmissions in Tg(MHu2M) mice, it was proposed that the most likely mediator of this inhibition is an auxiliary non-PrP molecule, provisionally designated protein X, which participates in the formation of prions by interacting with PrP^C to facilitate conversion to PrP^{Sc}.

With the aim of decreasing the incubation time of disease in Tg(MHu2M) mice, transgenes were constructed in which one or more of the nine human residues of the human insert of MHu2M

were changed to mouse. Mice expressing a double substitution of two C-terminal residues (M165V and E167Q), designated Tg22372 mice, which express the transgene at the 1-2 X level of wild type PrP, become ill with a reduced incubation time of 106 to 114 days after challenge with sporadic CJD prions.³⁶

Because the bioassay for bovine prions in wild-type mice is relatively insensitive,³⁷ and the expense and long bovine spongiform encephalopathy (BSE) incubation times in cattle make them unsuitable for bioassay studies, it seemed likely that transgenic approaches would offer a more accurate and convenient means of determining BSE titers. Based on the success of Tg(MHu2M) mice, transgenic mice expressing a similar chimeric mouse–bovine PrP construct, referred to as MBo2M, were produced. While transgenic mice expressing bovine PrP, referred to as Tg(BoPrP) mice, developed disease after inoculation with BSE, albeit with incubation times between 250 and 300 days, Tg(MBo2M) mice did not develop disease after challenge with BSE.³⁸ Similar results were independently obtained by another group,³⁹ and a recently developed bovine transgenic mouse model, created using the MoPrP.Xho expression vector and *Prnp*^{0/0} mice, was also susceptible to BSE prions.⁴⁰

Transgenic mice expressing a chimeric sheep/mouse or bovine/mouse PrP gene resembling the Tg(MHu2M) model have also been produced.⁴¹ Following intracerebral inoculation with three scrapie isolates, minimal amounts of sheep/mouse PrP converted to PrP^{Sc} with a disease incubation time of >500 days. No chimeric bovine/mouse PrP^{Sc} was detected, and these mice died between 600 and 700 days after inoculation. To address the issue that the wild-type allele could inhibit the conversion of chimeric PrP, the transgenes were also expressed on a *Prnp*^{0/0} background, which actually resulted in prolonged incubation times for the sheep chimeric mice and no transmission in bovine chimeric mice, again without evidence of conversion of chimeric PrP.

Transgenic mice expressing the ovine PrP coding sequence with alanine, arginine, and glutamine (ARQ) at codons 136, 154, and 171, under the control of a neuron-specific enolase promoter on a *Prnp*^{0/0} background, were developed to study transmission of natural sheep scrapie.^{42,43} Mice inoculated with two different scrapie strain isolates succumbed to disease with an incubation period of 238 to 290 days and developed clinical signs, spongiform changes, and accumulation of PK-resistant PrP^{Sc} in their brains, whereas nontransgenic mice remained scrapie free for more than 700 days. When inoculated with brain homogenate from sheep experimentally infected with BSE, these mice became ill within 300 days with prominent florid pathology as found in vCJD cases.⁴² Transgenic mice overexpressing ovine PrP with valine, arginine, and glutamine (VRQ) at codons 136, 154, and 171 under the control of *Prnp* regulatory sequences developed clinical illness ~70 days after inoculation with scrapie.⁴⁴

Transgenic Studies of Prion Strains

Because of their unprecedented mode of replication, explaining the mechanism by which prions propagate strain information posed a major challenge to the prion hypothesis. Two strains of transmissible mink encephalopathy (TME) produced different clinical symptoms and incubation periods in Syrian hamsters and showed different resistance to proteinase K digestion and altered amino-terminal proteinase K cleavage sites,⁴⁵ suggesting that different strains might represent different conformational states of PrP^{Sc}. Evidence supporting this concept emerged from transmission studies of inherited human prion diseases in transgenic mice. Expression of mutant prion proteins in patients with FFI or familial CJD (fCJD) results in variations in PrP conformation, reflected in altered proteinase K cleavage sites, which generate PrP^{Sc} molecules with molecular weights of 19 kDa in FFI and 21 kDa in fCJD(E200K).²⁹ Extracts from the brains of FFI and fCJD(E200K) patients transmitted disease to Tg(MHu2M) mice after about 200 days on first passage and induced formation of 19 kDa PrP^{Sc} and 21 kDa PrP^{Sc}, respectively. Upon second passage in Tg(MHu2M) mice, these characteristic molecular sizes remain constant but the incubation times for FFI and fCJD(E200K) prions diverge. These results indicate that PrP^{Sc} conformers function as

templates in directing the formation of nascent PrP^{Sc} and provide a mechanism to explain strains of prions where diversity is enciphered in the tertiary structure of PrP^{Sc}. The strain characteristics of fCJD caused by the V210I mutation were also defined following transmission to Tg(MHu2M) mice.⁴⁶

A sporadic form of fatal insomnia, referred to as sFI, has also been described.⁴⁷ Although patients with sFI have symptoms and neuropathological profiles indistinguishable from patients with FFI, they do not express the D178N mutant form of human PrP^C. sFI prions were transmitted to Tg(MHu2M) mice and were found to produce an identical pattern of neuropathology to that in Tg mice infected with FFI prions, arguing that PrP^{Sc} isoforms associated with sporadic fatal insomnia and fatal familial insomnia have the same conformation. These findings argue that the conformation of PrP^{Sc}, not the amino acid sequence, determines the strain-specified disease phenotype.

Other studies have shown that different sporadic and iatrogenic CJD cases associated with specific codon 129 genotypes can be typed according to PrP^{Sc} fragment sizes following PK treatment and Western blotting of brain extracts.⁴⁸ A characteristic banding pattern of PrP^{Sc} glycoforms found in vCJD patients and BSE-infected animals distinguishes vCJD PrP^{Sc} from the patterns observed in classical CJD.^{49,50} Transgenic mice expressing mutations at one or both glycosylation consensus sites have been studied to investigate the role of the asparagine-linked oligosaccharides of PrP.⁵¹ Mutation of the first site altered PrP^C trafficking and prevented infection with two prion strains; deletion of the second did not alter PrP^C trafficking, permitted infection with one prion strain, and altered the pattern of PrP^{Sc} deposition.

Initial transmission studies of human CJD cases in Tg mice were extended to include a larger number of additional sporadic cases. Transmission of these cases to Tg(MHu2M) and Tg(HuPrP) mice expressing either valine (V) or methionine (M) at codon 129 revealed that this polymorphism and the strain-specified conformation of PrP^{Sc} profoundly influenced the length of the incubation time and patterns of PrP^{Sc} deposition in recipient mice.^{11,36} Also, the size of the protease-resistant PrP^{Sc} fragment in human brains was reproduced on primary and secondary passages of vCJD, sCJD, fCJD(E200K), and FFI prions in Tg(MHu2M) mice. The constancy of the strain-specified tertiary structure on serial passage through Tg(MHu2M) mice contrasts with other parameters used to characterize prion strains, such as incubation periods and neuropathology profiles. Separate transgenic studies on the role of the codon 129 genotype in CJD transmission also showed that transgenic mice expressing human PrP M129 were more susceptible to sporadic CJD derived from M/M patients than CJD inocula from M/V or V/V patients.⁵² Transmission of BSE and vCJD to transgenic mice expressing human PrP 129M resulted in the neuropathological and molecular phenotype of vCJD. In addition to producing a vCJD-like phenotype, BSE transmission also resulted in a molecular phenotype indistinguishable from that of sporadic CJD, suggesting that more than one BSE-derived prion strain might infect humans.⁵²

Tg(MH2M) mice expressing chimeric hamster/mouse PrP were used to study the emergence of new prion strains by primary and secondary passage of SHa strains Sc237 and DY. Prion strains were defined by incubation time, neuropathological lesion profiles, and PrP^{Sc} conformation. Only Sc237 manifested a species barrier effect in Tg(MH2M) mice. Serial passage of brain homogenate from sick Tg(MH2M) mice inoculated with Sc237 into Tg(MH2M) mice resulted in a decreased incubation on second passage with a reproduction of the prominent GSS amyloid pathology observed in the first passage. The original Sc237 and the MH2M(Sc237) inocula were analyzed using a conformational stability assay, which assigns profiles to different prion strains based on the rate of PrP^{Sc} denaturation in the presence of guanidine hydrochloride (GdnHCl) prior to PK digestion. Interestingly, the [GdnHCl]_{1/2} values of SHa (Sc237) and TgMH2M(Sc237) inocula were significantly different, while the SHa (DY) and MH2M(DY), which lack a species barrier, displayed very similar conformational states based on these values.⁵³

Transgenic Studies of Prion Pathogenesis

It appears that accumulation of PrP^{Sc} may not be the sole cause of pathology in prion diseases, since certain examples of prion disease occur without accumulation of protease-resistant PrP^{Sc}.^{24,25} moreover, the time course of neurodegeneration is not equivalent to the time course of PrP^{Sc} accumulation in mice expressing low levels of PrP^C.⁵⁴ An alternative mechanism of PrP-induced neurodegeneration was suggested by transgenic studies of mutant forms of PrP that disrupt PrP biogenesis in the endoplasmic reticulum.⁵⁵ Transgenic mice expressing mutations in the stop transfer effector region between residues Lys 104 and Met 112 and the hydrophobic TM1 region between residues Ala113 and Ser135 spontaneously develop neurodegenerative disease and accumulate an aberrant form of PrP termed ^{Ctm}PrP, which appears to be different from conventional protease-resistant PrP^{Sc}. Accumulation of ^{Ctm}PrP is also associated with a form of GSS that segregates with the codon 117 mutation of *PRNP*.

The role of alternatively processed forms of PrP in prion pathogenesis is currently a topic of debate. Recent studies have focused on the role of a novel cytosolic form of PrP in neurodegeneration. Proteasome inhibition resulted in retrograde transport of PrP from the endoplasmic reticulum and cytoplasmic accumulation of a PrP^{Sc}-like form that was apparently self-perpetuating.⁵⁶ Transgenic mice expressing cytosolic PrP presented with ataxia as early as 7 weeks of age.⁵⁷ The neuropathology of these mice consisted of cerebellar atrophy, neuronal loss in the granular and molecular layers, and severe gliosis in the cerebellum. PK-resistant PrP^{Sc} was not detected. The authors proposed that circumstances where proteasomal function is either diminished, such as in normal aging, or overwhelmed, such as in familial prion diseases where point mutations could initiate PrP misfolding, result in propagation of cytosolic PrP and accumulation into amorphous aggregates that are responsible for neuronal damage. It is currently not known whether cytosolic PrP is infectious. Subsequent studies in primary human and mouse cerebellar granular neurons demonstrated that cytosolic PrP is not necessarily neurotoxic.^{58,59}

ECTOPIC EXPRESSION STUDIES

Although the pathological consequences of prion infection occur in the CNS, PrP^C has a wide tissue distribution, and the exact cell types responsible for agent propagation and pathogenesis are still uncertain. Next to brain, lung tissue has the highest level of expression, and PrP^C is detectable on the surfaces of lymphocytes and in heart, skeletal muscle, intestinal tract, spleen, testis, ovary and some other organs. In the CNS, PrP is expressed in neurons throughout the life of the animal, with levels of PrP mRNA varying among different types of neurons.⁶⁰ PrP mRNA is also expressed in astrocytes and oligodendrocytes throughout the brains of postnatal hamsters and rats.⁶¹ The level of glial PrP mRNA expression in neonatal animals is comparable to that of neurons and increases twofold during postnatal development.

Using different gene control elements, it has been possible to direct the expression of PrP to specific cell types. While previous reports found little or no prion infectivity in skeletal muscle, two types of transgenic mice in which expression of PrP^C is directed exclusively to muscle under the control of the muscle creatine kinase and chicken α -actin promoters demonstrated that this tissue is capable of propagating prion infectivity.⁶² Transgenic mice in which expression of SHaPrP was regulated by the neuron-specific enolase promoter indicated that neuron-specific expression of PrP^C was sufficient to mediate susceptibility to hamster scrapie.⁶³ Interestingly, astrocytes have been found to be the earliest site of PrP^{Sc} accumulation in the brain,⁶⁴ suggesting that these cells may play an important role in scrapie propagation and/or pathogenesis or even that astrocytes themselves may be the cells in which prion propagation occurs. Transgenic mice expressing hamster PrP under the control of the astrocyte-specific glial fibrillary acidic protein (GFAP) accumulated infectivity and PrP^{Sc} to high levels and developed disease after ~220 days.⁶⁵ The interferon

regulatory factor-1 promoter/E μ enhancer, Lck promoter, and albumin promoter/enhancer have been used to direct PrP expression to the spleen, T lymphocytes, and liver respectively.⁶⁶ High prion titers were found in the spleens of inoculated transgenic mice expressing PrP under the control of the interferon regulatory factor-1 promoter/E μ enhancer, while mice expressing PrP under the control of the Lck and albumin promoters failed to replicate prions.

Knockout Mouse Models

Some of the most compelling evidence to date for the so-called “protein-only hypothesis” of prion replication derives from experiments with knockout transgenic mice. Since PrP^C is the source of PrP^{Sc}, the model predicted that elimination of PrP^C would abolish prion replication. To test this, the mouse PrP gene, referred to as *Prnp*, was disrupted by homologous recombination with a genetically modified version in embryonic stem cells. Stem cells containing the disrupted *Prnp* gene were introduced into mouse blastocysts, and knockout mice were established.^{67–69} Unlike wild-type mice, the resultant homozygous null mice (*Prnp*^{0/0}) fail to develop the characteristic clinical and neuropathological symptoms of scrapie after inoculation with mouse prions and do not propagate prion infectivity,^{69–72} while mice that are hemizygous for PrP gene ablation have prolonged incubation times.^{54,71,72}

Since *Prnp*^{0/0} mice developed normally and suffered no gross phenotypic defects, this raised the possibility that adaptive changes occur during the development of *Prnp*^{0/0} mice that compensate for the loss of PrP^C function. To test this hypothesis, transgenic mice were produced in which expression of transgene-expressed PrP^C could be controlled at will. Using the tetracycline gene-response system, mice were produced that coexpress a tetracycline-responsive transactivator, referred to as tTA, and a tTA-responsive promoter that drives PrP expression.⁷³ Repressing PrP^C expression by oral administration of doxycycline was not deleterious to adult mice. However, since doxycycline treatment did not completely inhibit PrP^C expression in these mice, it is not clear whether this residual expression masks the true phenotype of *Prnp*^{0/0} mice.

An alternative approach to creating a conditional postnatal PrP knockout utilized a Cre-loxP system.⁷⁴ Double transgenic mice were produced by crossing mice in which the PrP gene is flanked by lox-P sites with mice in which Cre recombinase is expressed in neurons under the control of the neurofilament promoter. Double transgenic offspring, referred to as NFH-Cre mice, express PrP up until 10 weeks of age, when adult-onset activation of the neurofilament-controlled Cre recombinase occurs. Mice remained free of any abnormal physical phenotype up to 15 months postknockout but had decreased afterhyperpolarization potentials in hippocampal CA1 neurons, suggestive of an electrophysiological function of PrP, perhaps associated with Fyn-mediated PrP neuronal signaling.⁷⁵ These data are in support of other studies that demonstrate impairment of GABA_A receptor-mediated fast inhibition and long-term potentiation in hippocampal slices from *Prnp*^{0/0} mice.^{76,77} Several other phenotypic defects have also been reported in *Prnp*^{0/0} mice, including altered circadian rhythms and sleep patterns,⁷⁸ alterations in superoxide dismutase activity (SOD-1),⁷⁹ and defects in copper metabolism.⁸⁰

Recently, Malluci and colleagues demonstrated that brain lesions associated with terminal prion disease are reversible.⁸¹ Transgenic NFH-Cre mice were inoculated with prions at 3 to 4 weeks of age. At 10 to 12 weeks of age, at which point the brains of infected mice had accumulated significant extraneuronal PrP^{Sc} deposits and exhibited spongiform pathology, Cre recombinase initiated a recombination of the lox sites flanking the PrP gene resulting in elimination of neuronal PrP expression. Mice were allowed to age after this process and were ultimately rescued from the terminal phase of prion disease. The brains of the aged showed reversal of the spongiform pathology but still harbored PrP^{Sc} deposits in nonneuronal cells, in which Cre-mediated recombination did not occur.

While *Prnp*^{0/0} mice independently created in Zurich and Edinburgh developed normally and showed no overt phenotypic defects, a third line of gene-targeted *Prnp*^{0/0} mice generated in Nagasaki

showed progressive ataxia and cerebellar Purkinje cell degeneration at about 70 weeks.⁸² In addition to the PrP coding sequence, ~900 nucleotides from the second intron and 450 nucleotides of the 3' noncoding sequence of *Prnp* are deleted in these mice. The molecular basis of the phenotypic differences in these *Prnp*^{0/0} lines is derived from the discovery of a PrP-like gene that encodes the Doppel protein (Dpl), an acronym for downstream prion protein-like gene (*Prnd*) located 16 kb downstream of *Prnp* in the murine genome and 27 kb downstream of human *Prnp*.^{83,84} Discovery of *Prnd* led to the notion of a prion gene complex that encodes PrP, Dpl, and a more recently identified third *Prnt* gene located 3 kb on the 3' downstream of *Prnd*.⁸³

Doppel has 24% sequence homology and shares many structural similarities with PrP^C: both proteins are GPI-anchored to the external face of the plasma membrane and have two *N*-glycosylation sites and a globular C-terminus with three alpha-helices and two short strand beta-strands and a disulfide bond.⁸⁵ Additionally, both are copper-binding proteins, despite the lack of the octapeptide repeats in the *N*-terminus of Doppel.⁸⁶ Doppel is primarily expressed by spermatid cells in the testis and is absent in the CNS.⁸⁵ Interestingly, *Prnd* knockout mice are sterile.⁸⁷ In certain *Prnp*^{0/0} mouse models, Doppel is expressed in the CNS as the result of an alternative intergenic splicing event ensuing from the different molecular strategies used to disrupt the PrP gene.^{84,88} This unnatural expression of Doppel in the CNS led to late-onset ataxia and Purkinje cell loss and the discovery of Doppel underlying neurotoxic effects in the absence of PrP^C expression.

Correlation of Doppel expression with a neurological phenotype resembling clinical prion disease suggested that Doppel could play a key role in prion pathogenicity. However, reintroduction of wild-type PrP resulted in offspring lacking an ataxic phenotype.⁸⁹ Another knockout line, referred to as Zurich II,⁸⁸ in which the entire PrP-encoding exon and its flanking regions are recombined between lox P sites, was created to further address this issue. Zurich II mice express Doppel and develop ataxia and when crossed to Zurich I mice, heterozygous offspring expressed half as much Doppel as Zurich II mice and had a 6-month delay in appearance of ataxia. Introduction of a single wild-type PrP allele blocked the ataxic phenotype of Zurich II mice, indicating that PrP plays a role in protecting against Doppel-induced neurotoxicity. To further investigate the protective mechanism of PrP from Doppel-induced toxicity, various PrP transgenes were introduced into Nagasaki *Prnp*^{0/0} mice.⁹⁰ All transgenes except for one construct lacking amino acid residues 23 to 88 rescued the ataxic phenotype and Purkinje cell death. Expression of *N*-terminally truncated PrP directed to Purkinje cells of Zurich I mice, which normally remain healthy, also resulted in ataxia and Purkinje cell loss.¹⁸ Again, introduction of a single PrP allele rescued this phenotype.

GENE TARGETING APPROACHES

Gene targeting is a technique in which the wild type PrP gene (referred to as *Prnp*) is precisely replaced with an altered transgene by homologous recombination in embryonic stem cells, allowing for production of transgenic mice that express altered PrP molecules at normal levels under endogenous transcriptional controls. This approach eliminates potential complications arising from microinjection of linearized DNA constructs including transgene integration effects, increased levels of transgene expression, and altered tissue-specific expression.

Gene targeting has been used to definitively address the role of *Prnp* polymorphisms associated with long and short scrapie incubation time alleles. Inbred strains of mice with long and short scrapie incubation periods harbor distinct *Prnp* alleles, referred to as *Prnp*^a and *Prnp*^b, which differ at codons 108 (Leu to Phe) and 189 (Thr to Val).⁹¹ To precisely define the effects of these PrP polymorphisms on prion incubation times, the coding sequence of the endogenous *Prnp*^a gene in embryonic stem cells was replaced with the *Prnp*^b coding sequence by homologous recombination. Studies with the resulting transgenic mice confirmed, as predicted, that *Prni* and *Prnp* are the same gene and that amino acid differences at residues 108 and/or 189 in PrP-A and PrP-B modulate scrapie incubation times.⁹²

A mutation equivalent to P102L in the human PrP gene, associated with GSS, has also been introduced into *Prnp* by gene targeting.⁹³ Homozygous gene-targeted mice, designated 101LL, express the mutant protein at wild-type levels and did not develop spontaneous prion disease during the course of their lifespan. Moreover, inoculation of wild type and 101LL mice with brain extract from aged 101LL mice did not transmit disease. When inoculated with prions from human GSS patients with the P102L mutation, hamster 263K prions, or SSBP/1 sheep scrapie, but not with human vCJD, 101LL mice developed neurodegenerative disease more rapidly than did wild-type recipients, indicating that the 101L mutation influences incubation time in a strain-specific manner.⁹⁴

Following the success of the Tg(MHu2M) model, mice expressing chimeric human/mouse PrP, referred to as (Ki-ChM), were created using a knock-in strategy in which the mouse PrP gene was replaced with a PrP transgene containing human amino acids from residues 23 to 188 and murine amino acids from 189 to 231.⁹⁵ Following inoculation with sporadic, familial, or iatrogenic human CJD brain homogenates, (Ki-ChM) mice succumbed to disease with incubation times between 151 and 200 days. Seventy-five days post inoculation, follicular dendritic cells (FDC) from all lymphoid organs contained converted chimeric PrP^{Sc}, with the splenic FDC proving infectious upon serial transmission. This model holds promise as a relatively rapid bioassay for human prions.

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Animal Models for the Study of Allergy

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INTRODUCTION

Allergy results from the recognition by the immune system of, and heightened reactivity toward, what are normally innocuous materials in the environment. Under normal circumstances, soluble proteins in contact with the mucosal surfaces do not provoke strong immune responses but instead induce a state of antigen-specific hyporesponsiveness. However, under other circumstances, the cause of which is yet unknown in many cases, such proteins induce allergic inflammatory responses.¹ By definition, allergy represents an adverse immune response manifested by an IgE antibody-dependent (atopic) recognition of proteins that can result in a number of disease manifestations. Allergic diseases represent complex events that are characterized by inflammatory responses consisting of Th2 lymphocytes, elevated specific-IgE, eosinophils and basophils; however, mechanisms are less well understood. Adverse effects develop in two stages:

1. Sensitization, requiring a sufficient or cumulative exposure dose of the sensitizing agent to induce an IgE immune response
2. The allergic response, which occurs in sensitized individuals upon subsequent exposure to the inducing agent

Emphasis is placed upon antigen/allergen-induced immunoregulation and the resulting factors, such as cytokines, chemokines, pathway-activating factors, and inflammatory and remodeling factors, that contribute to the respective diseases.

The pathogenesis of allergies, which includes skin (atopic dermatitis, contact dermatitis), ocular (allergic conjunctivitis), respiratory (hay fever and asthma), and gastrointestinal allergies (food allergy), involves a multiplicity of symptoms that are difficult to evaluate by *in vitro* procedures. Mice, rats, and guinea pigs continue to be the focus of research on gastrointestinal and respiratory allergies, with recent contributions of domestic animals, such as dogs, swine, and bovine making contributions to scientific scrutiny. The focus of this chapter will be on these animals and the contributions research investigations have provided in the areas of respiratory and gastrointestinal allergies (food allergy) and atopic dermatitis. Put into perspective, Hein and Griebel² suggest that basic advances in immunological knowledge from animal models, small or large, should translate to useful clinical outcomes. In that context, the physiological relevance of animal models selected should be a key factor in how efficiently a laboratory “proof of concept” can be translated to successful clinical treatments. Cost, convenience of handling, and immunological reagents used as tools available for intended research designs have led to the predominant use of rodent models in immunological research. However, large-animal models have unique experimental advantages, for example, closer immunophysiology to humans, as well as natural diseases that can contribute to the pathogenesis of human allergic diseases.

As in humans, animal models have an innate tendency to develop tolerance to the myriad of proteins ingested, and it is difficult to generate valid food allergy models. The degree of sensitization should take into consideration the following parameters:

1. The concentration of the allergen (High doses are known to induce tolerance; however, the high-dose tolerance/low-dose sensitization is relative to the host and the antigen/allergen source.)
2. The need to take the allergen in context with the food source
3. The route (feeding and/or gavage are the recommended avenues) and duration (time course may vary with respect to species and allergen) of allergen exposure
4. The age of the animal (neonate, adolescent, adult)

Other considerations include:

5. Genetic predisposition (high and low IgE responders)
6. Use of adjuvants (natural or artificial — cholera toxin, *Bordetella pertussis*, and carrageenan are known IgE-selective adjuvants)
7. Isotype specificity response (Mice respond with two anaphylactic antibodies, IgG1 and IgE; rats with IgG2a and IgE; guinea pigs with IgG1 and IgE; dogs with IgE; pigs, likely with IgE.)
8. Th1/Th2 regulation/polarization (Mice have very delineated Th1/Th2 polarization, whereas in the human, polarization is not as discrete.)

An example of animal interpretation is the C3H/HeJ mouse strain, which has a point mutation in Toll-like receptors that underlies a defect in the lipopolysaccharide (LPS)-induced cytokine production by peritoneal macrophages.³ Knockouts, transgenic models and species of the model under investigation contribute a great deal of information to the mechanistic and therapeutic options; however, the results must be taken into context when extrapolating to human allergic diseases.

ATOPIC DERMATITIS

Allergic contact dermatitis is a cutaneous inflammatory reaction mediated by antigen-specific immune-competent cells. The disease develops as a result of a complex interaction of environmental exposure, genetic background, and immunologic factors. Although allergic contact dermatitis and

contact hypersensitivity models have been documented as Th1-predominant immune responses, Wang et al.⁴ clearly demonstrated that epicutaneous administration of hapten through patch application could modulate the balance between Th1 and Th2 responses by continuous administration of protein allergen. This model could contribute to the regulatory mechanisms concerning the role of antigen and administration route for contact hypersensitivity.

NC mice, which develop spontaneous dermatitis at or near weaning, present with clinical symptoms and IgE hyperproduction that could be influenced by environmental factors.^{5,6} These mice could be used for determining a natural atopic dermatitis associated with skin barrier dysfunction and histopathology that closely resembles this form of atopic dermatitis. Murine models of atopic dermatitis include the NC/Nga strain, which have been proposed; however, the trigger appears to be environmental and not related to food.⁷ Under ambient air conditions, biophysical measurement indicated barrier-perturbed skin, a significant decrease in ceramide content (measurement of water retention and skin barrier function), and marked clinical and histological conditions elucidating aspects of atopic dermatitis pathogenesis. About one-third of female C3H/HeJ mice sensitized to cow's milk or peanut develop eczematous eruptions with eosinophilia, elevated IgE levels, and cytokine involvement that suggest this model may truly represent food hypersensitivity and provide immunopathogenic mechanistic studies that extrapolate to human disease.⁸

Results of a murine model of atopic dermatitis established a role for Th2 cytokines, IL-4 and IL-5, as well as the Th1 cytokine INF-gamma.⁹ IL-4 and IL-5 were shown to be important for eosinophilic infiltration, whereas IL-5 and INF-gamma were important for skin hypertrophy. Modulation of the Th1/Th2 cytokine balance may be a significant factor in both the presentation of atopic dermatitis and in therapeutic strategies for treatment. Results from the same group using epicutaneous sensitization with protein antigen led to local allergic dermatitis and antigen-specific airway responsiveness upon airway challenge to the same antigen.¹⁰ This suggests that prolonged epicutaneous sensitization may have a significant effect upon allergic asthma with this antigen. More studies will need to be performed to support skin sensitization with different antigens that could progress to airway disease.

A review of the similarities existing between companion and laboratory animals and the human counterpart to atopic dermatitis is presented by Marsella and Olivry.¹¹ Spontaneous atopic dermatitis in canine, feline, and murine models is highlighted against the background human disease. Nuttall et al.,¹² using reverse transcriptase-polymerase chain reaction (RT-PCR) to identify cytokines in both atopic and healthy skin of dogs recruited from veterinary dermatology clinics, demonstrated that atopic dermatitis was associated with increased levels of IL-4, while clinical tolerance was suggested to be associated with TGF-beta. The pathogenic sequence of early cytokine profiles (Th2) and lesional skin, which showed a mixed cytokine profile, was discussed while recognizing the fact that mechanisms are still unclear for the respective presentations of this naturally occurring model of human atopic dermatitis. Future investigations in the naturally occurring model of atopic dermatitis in canines with similar characteristics of a Th1/Th2 polarization should lead to better treatment and care for applications to human atopic dermatitis associated with environmental allergens.

Naturally occurring allergic skin disease in dogs is similar to IgE-dependent food-related atopic dermatitis with primed effector cells distinguishing atopic from nonatopic conditions. Immunohistochemistry studies of skin biopsies from atopic and nonatopic dogs revealed an obvious difference in stem cell factor that led the authors to suggest that higher levels of stem cell factor production in the skin of atopic dogs was the cause of hyperexcitability of skin mast cells.¹³ In a subsequent paper, canine atopic dermatitis was shown to be associated with more variability in circulating monocytes and lower numbers of monocytes expressing IgE.¹⁴ A significant difference in this model and human reports is that total serum IgE concentrations did not correlate with cell-bound IgE. Although not fully elucidated, experimental models of induced food allergy suggest that dogs with IgE-mediated food allergy respond in a histopathologically similar way to that seen in human atopic dermatitis.

OCULAR ALLERGY

Ocular allergy presents as an immunopathological reaction of the ocular surfaces that come into contact with the external environment. It represents a complex disease that spans seasonal allergic conjunctivitis to sight-threatening keratoconjunctivitis and vernal keratoconjunctivitis.¹⁵ Allergic conjunctivitis predominates in the literature with mice, rats, guinea pigs, rabbits, and dogs listed as animal models. Bundoc and Keane-Myers¹⁶ have provided a table identifying agents, parameters measured, outcomes, and species limitations, concluding there is a paucity in the studies of ocular allergy. Insights into pathogenesis of ocular allergy were noted, which included cytokines, adhesion molecules, and eosinophilic infiltration as well as different modes of therapeutic intervention. Although the cellular and molecular basis of this disease was considered to be complex, further research into the basic immunopathology using transgenic and knockout mice was recommended. Calonge et al.¹⁷ reviewed the animal models of ocular allergy, emphasizing mechanistic and therapeutic studies and selection of appropriate animal models to further investigations into etiopathogenesis and therapeutic treatment in experimental models of chronic ocular allergy. Notable in this review was the call for models using natural routes of allergen exposure, natural allergens, and appropriate tissue sites (conjunctival and nasal mucosa) that more closely resemble human allergic diseases affecting the eye. Both groups clearly emphasized the need for additional animal model studies to further the understanding of clinical correlates to human ocular allergy, in particular chronic forms of allergy.

Several murine models that mimic human allergic conjunctivitis are contributing to trait characterization of ocular allergy. The role of histamine receptors in late-phase reactions of allergic conjunctivitis (wild-type and H1-receptor-deficient mice) suggests that vascular permeability in the conjunctiva is entirely regulated by the histamine H1 receptor.^{18,19} Topical challenge of allergen in ragweed-sensitized wild type, IL-4 knockout (IL-4KO), IL-12KO, IFN-gamma KO and recombinant anticytokine KOs suggested that the presence of IL-12 was important in both the development and regulation of late-phase pathologic features in ocular allergy.²⁰ Thus, a Th1-type response may be important in the development of late-phase ocular allergy. More investigations are required to determine the role of Th1/Th2 balance in this model. In a related article, Magone et al.²¹ demonstrated that systemic and topical administration of immunostimulatory oligonucleotides (ISS-ODN) could provide antiinflammatory and immunomodulatory effects in the ragweed murine model of allergic conjunctivitis. As in other hypersensitivity diseases, this alternative modality of treatment using immunostimulatory agents may prove highly useful.

With increased visual tasking, an increased awareness of ocular allergy and its impact on quality of life has made a significant impact. Abelson and Loeffler²² have reviewed the science behind allergen challenge in the eye, calling for increased awareness in model design and the use of conjunctival allergen challenge in the treatment of ocular allergy. Treatment with novel recombinant peptides (antiflammins) that coinhibit phospholipase A₂ and transglutaminase 2, active in the arachidonic acid cascade of inflammation, clearly reduced the symptoms of ragweed-induced allergic conjunctivitis in guinea pigs, suggesting a more spectral use of these peptides for antiallergic and antiinflammatory drugs in other inflammatory diseases.²³ A reported reliable, economical, rapid, easy-to-perform murine model that mimics human allergic conjunctivitis using airborne allergen, and nasal and conjunctival mucosa exposure with no adjuvants resulted in the animal demonstrating high levels of antiragweed anti-IgE with clinical and histologic signs of allergic conjunctivitis.²⁴ Moreover, the conjunctivitis could be modulated by necrodisodium, a well-known antiallergy drug, suggesting this model could be used for studies of disease pathophysiology and treatment responses. Treatment regimens are significant in this area of allergy, while mechanistic studies still appear to be secondary.

RESPIRATORY ALLERGY

Aerosolized particles can lead to allergic reactions in the lung either directly as the allergen source or indirectly by enhancing allergic sensitization or exacerbation of allergic symptoms. The mouse is at the forefront in literature citations regarding *in vivo* animal models; however, it does not represent a natural model and should be considered only for dynamics of certain traits of the asthma phenotype and not the entire allergy/asthma phenotype in humans.²⁵ Miyajima et al.,²⁶ using BALB/c mice with targeted mutations of either the Fc-epsilon receptor (FcR) or Fc-gammaRI, suggested that mortality associated with active anaphylaxis was dependent upon the FcR gamma chain and not the FcR epsilon chain. The authors further concluded that the pathophysiology of active anaphylaxis was neither solely dependent upon signaling by FcR in mice nor could they conclude that IgE and mast cells did not have a contributory role in active anaphylaxis. In discussing the mechanisms of allergic inflammation underlying asthma, Kumar and Foster²⁷ suggested that short-term models do not represent the human asthma pathophysiology and attention should be given to modeling lesions of chronic asthma. To examine this chronic state, BALB/c mice were sensitized to aerosolized ovalbumin (OVA) in a whole-body inhalation exposure chamber for a period of 8 weeks. Responding animals exhibited progressively increasing airway-specific acute-on-chronic inflammation of the lung with airway hypersensitivity. In a mouse model of chronic asthma, responses in wild-type mice were compared to mice lacking both IL-4 and IL-13, as well as mice lacking signal transducer and activator of transcription 6 (STAT6), a key pathway for signaling of these two cytokines.²⁸ IL-4/IL-13 double knockouts exhibited little recruitment of eosinophils into the airway epithelium and no airway hyperresponsiveness. Both inflammation and airway hyperresponsiveness were undiminished in the STAT6-deficient mice. The results of this chronic asthma model suggest differences in short-term studies with the pathobiology of chronic asthma being significantly different. Although these findings implicate Th2 cytokines, inhibition of their signaling pathway through STAT6 is less likely to be a successful therapeutic option. Although limitations in interpretation are inherent in these models, murine models can contribute valuable information on some specific traits of allergy pathogenesis and therapeutic options that may well extrapolate to a clinical setting.

Exposure to allergens of *Aspergillus fumigatus* results in human lung diseases that include allergic asthma, hypersensitivity pneumonitis, and allergic bronchopulmonary aspergillosis (ABPA). Exposure to antigens of this mold in mice leads to allergy very similar to ABPA with high IgE levels, elevated numbers of peripheral blood and lung eosinophils, pulmonary inflammation, and airway reactivity including cytokine and chemokine involvement.²⁹ Mice receiving *A. fumigatus* antigens by intraperitoneal, subcutaneous, or nasal challenges were again challenged with *A. fumigatus* conidia via the intratracheal route.³⁰ The sensitized mice responded with chronic airway changes including airway hyperresponsiveness, peribronchial fibrosis, and mucus hyperplasia with significant increases in IFN-gamma, IL-4, TGF-beta, and IL-13. In a subsequent study, IL-18 was shown to modulate the innate immune response (Toll-like receptor expression) preventing development of severe fungus-induced asthmatic disease.³¹ The chronic nature of the response was shown to be IL-13 dependent but not STAT6 independent,³² and the persistence of the asthmatic disease was suggested to be associated with the chemokine receptor CXCR2.³³ The model will provide examination into the various aspects of fungal chronic allergy, paying particular attention to airway remodeling and the involvement of Th1/Th2 cytokines and receptors.

Two animal models of allergy, the pollen-allergic brown Norway (BN) rat and the ovalbumin BALB/c mouse, have been used to compare the discriminatory value of these two models to evaluate particulate material in the European Respiratory Allergy and Inflammation Due to Ambient Particles study.³⁴ Using diesel exhaust particles, residual oil fly ash, Ottawa dust, and road tunnel dust as source material during sensitization or challenge phases, the ovalbumin mouse model proved to be the most sensitive model to detect adjuvant effects of airborne particles. In BN rats exposed to a combination of pollens and particulate matter, the percentages of eosinophilic granulocytes were

decreased compared to BN rats immunized with pollen alone. In mice, IgE levels in serum were increased after coexposure to ovalbumin and particulate material in the sensitization phase but not after coexposure during the challenge phase. The authors concluded that the murine model was a more sensitive system to detect adjuvant activity of airborne particles, whereas the pollen-induced rat model was less sensitive. Consideration of the animal model and allergen source was critical to the aims of this study. Irritant/adjuvant effects clearly played a role in the mouse model during sensitization.

The allergenicity of cow's milk proteins has been assessed in a guinea pig model. In this study, the allergenicity of milk-based infant formula and cow's milk proteins was evaluated by examining altered intestinal permeability, intestinal anaphylaxis, and passive cutaneous anaphylaxis after oral sensitization with cow's milk proteins and challenge with β -lactoglobulin.³⁵ Colonic segments from cow's milk-sensitized or milk formula-sensitized animals were challenged with β -lactoglobulin in Ussing chambers. Cow's milk-sensitized animals responded with an antigen-induced, anaphylactically mediated elevation in the transmural short circuit current as measured by net chloride secretion, whereas only 60% of animals fed infant formula responded to challenge. Bronchospasm developed in all animals fed cow's milk; however, only those animals fed infant formula that responded to intestinal challenge developed bronchospasm. The authors concluded that cow's milk-based infant formula had less sensitizing power than whole cow's milk and that the model was effective in testing allergenicity at the intestinal level. Both the isotype antibody responses (anaphylactic IgG1 and IgE are produced) and the immune system of the guinea pig, which has yet to be completely characterized, differ from that of the human.

Rat strains, including PVG, hooded Lister, and brown Norway, have been utilized to produce IgE antibody to food proteins as well as inhaled allergens. Atkinson et al.³⁶ showed that the IgE antibody responses in the brown Norway rat to allergens present in milk were similar to those identified in humans. In later studies, the toxicological concept of dose response was applied to immunological phenomena enabling a ranking of food proteins (ovalbumin and milk proteins) in terms of inherent allergenic potential.^{37,38} Plant products, carrageenan and saponins, were suggested to be adjuvants that altered allergen uptake across the intestinal mucosa.³⁹ Observations that constituents other than the allergens identified in food matrices, i.e., the fat emulsion of milk, may be responsible for inducing allergenic responses were made using milk-based immunization regimens consisting of individual allergenic proteins and peptide sequences versus milk.⁴⁰ The significant finding from this group is that the BN model recognized similar IgE-binding epitopes in β -lactoglobulin to that of serum IgE from milk-allergic patients. Comparisons of IgE and IgG recognition of β -lactoglobulin suggested that allergenic sites of β -lactoglobulin did not always correspond with antigenic determinants.⁴¹ Caveats include the use of carrageenan as an IgE-selective adjuvant and the route of intraperitoneal sensitization. Oral sensitization in BN rats to various allergenic food proteins in the absence of adjuvant resulted in antigen-specific IgE, local and systemic immune responses, and decreased respiratory function and blood pressure.^{42,43}

In these studies, daily gavage with hen's egg white, ovalbumin, and cow's milk over a 6-week period in the absence of adjuvant resulted not only in allergen-specific IgE, but also in the appearance of local and systemic immune-mediated effects following oral challenge. Immunoblotting experiments with serum obtained from BN rats orally sensitized to hen's egg white and cow's milk revealed antibody recognition profiles similar to that identified using serum obtained from humans with the same food allergy.⁴⁴ Pathophysiologic and inflammatory responses of the acute and late phase of allergic reactions in horseradish peroxidase-sensitized and orally challenged Sprague-Dawley rats revealed abnormal jejunal mucosa.⁴⁵ Mononuclear cells increased dramatically, and by 48 hours the cell number had increased approximately tenfold over that of controls, accompanied by altered mucosa with swelling of the villi, obvious edema, detachment of epithelial cells from the underlying tissue, and enhanced permeability for at least 72 hours.

Using Ccs/DEM-5, Ccs/DEM-11, Ccs/DEM-12, and Ccs/DEM-18 mice carrying random sets of 12.5% of genes from a Th1-responder STS strain and 87.5% of genes from Th2 BALB/c mice

for genetic mapping, studies showed different asthma traits; the presence of IgE, eosinophilia, and hyperresponsiveness could be identified.⁴⁶ These strains should prove valuable in the genetic mapping of specific allergic asthma traits. Despite the advantages of murine animal models of asthma, allergic lung diseases in dogs more accurately model the human condition.^{47,48} Research in Bice's laboratory using the ragweed-sensitized dog model to determine the role of air pollution in exacerbations of asthma symptoms has demonstrated the usefulness of the allergic dog model for investigations of underlying pulmonary immune mechanisms and therapeutic treatment of allergic asthma.^{49,50} A high IgE-producing inbred dog colony originally used for airway hyperresponsiveness to ragweed⁵¹ has subsequently been successfully sensitized and challenged with wheat,⁵² milk,⁵³ peanuts, and tree nuts.⁵⁴ This model represents a natural allergy model that can be used for airway and food allergy studies as well as a potential screening tool for assessing allergenicity of novel proteins introduced into genetically modified foods.

Although asthma appears to be restricted to the human species, animal models continue to be used to investigate particular aspects of this human disease. Isenberg-Fieg et al.⁵⁵ review, support, and criticize the use of various animal models in the study of allergic airway disease, including rodents and primates. They conclude that no animal model of asthma exactly reproduces the pathological effect or clinically serves as an adequate model of airway disease in humans. However, the contributions of animal model studies provided an understanding of the detailed pathogenesis and development of novel therapies to respirable allergens.

Murine models of asthma routinely involve relatively short-term sensitization and inhaled challenge similar to allergic responses rather than chronic asthma and vary with the strain under investigation.⁵⁶ Hegele⁵⁷ reviewed the role of viruses in the onset of asthma and allergy characterized by animal studies under the headings of (1) chronic sequelae of acute viral infections, (2) enhanced sensitization to aeroallergens, and (3) persistent viral infections. A great deal of information concerning cytokine involvement comes from the use of knockout models that support the Th2 hypothesis; however, differences in data are often attributed to differences in murine strains and experimental protocols. An example appears in studies of the contributions of IL-4 and IL-5 in airway hyperactivity.^{58,59} Despite pronounced inhibition of antigen-induced airway eosinophilia in mice treated with anti-IL-5, the increase in airway hyperresponsiveness was not prevented in these studies. However, Foster and colleagues convincingly showed that both allergen-induced eosinophilia and airway hyperactivity could be abolished in IL-5 knockout mice.⁶⁰ Sugita et al.⁶¹ discussed the role of these animal models in clinical applications and confirmed that a causal relationship between inflammation and hyperresponsiveness of the airway needs additional clarification. Recommendations to consider differences in the relationship between airway eosinophilia and hyperresponsiveness were based upon discrepancies identified as follows: interstrain, protocol, and route of administration differences and other differences noted in this review.

An overview of the possible interactions among mast cells, Th2 cells, eosinophils, airway remodeling, airway hyperactivity, acute bronchospasm, chronic asthma, and the T-cell-related cytokines in asthma was presented by Larche et al.⁶² The authors identified the following important questions to be addressed in future therapy studies:

1. Will direct targeting of T cells, such as through peptide therapy, be effective for chronic asthma, or does airway remodeling preclude major responses to T-cell-directed treatment?
2. What drives the T-cell response in nonallergic intrinsic asthma?
3. Are non-Th2 products important in T-cell-dependent airway narrowing?
4. What controls the activation of airway T cells in atopic nonasthmatic patients and healthy individuals?
5. Can treatments be developed that target multiple cytokines yet remain more specific than corticosteroids?
6. What chemokines and receptors are important in T-cell recruitment and retention in the asthmatic airway?⁶²

Thus, although the role of T lymphocytes and T-cell-related cytokines reinforces the intracellular control of T cells in the molecular mechanism for the pathogenesis of asthma, a considerable amount of research is still needed to determine mechanisms and treatment of asthma.

Sensitization, cross-reactivity, and clinical responses to food and pollen allergens are still controversial subjects. Studies indicate that mice immunized with grass pollen — with or without subsequent latex immunization — present with different clinical entities.⁶³ In a latex–pollen investigation, preimmunization with timothy pollen extract enhanced the production of IgE antilatelx antibodies, and latex sensitization maintained higher levels of IgE antibodies against pollen allergens in BALB/c mice. Cross-reactive antibodies were mutually enhanced *in vivo* with boosting of pollen and latex sensitization suggesting that polysensitization may occur in latex–pollen syndromes. The clinical responses may be associated with continued exposure to one or the other allergen and thus explain the different clinical responses in animals with IgE to both allergen sources. Artificial epitopes identified as mimotopes suggest that resistance to digestion may contribute to the modulation of Th1/Th2 responses in the gastrointestinal tract.⁶⁴ Studies such as these will go a long way in determining the clinical versus serological response to different allergens, exposure routes, and dose effects for type I allergic reactions.

Using 3-week-old C3H/HeJ mice fed homogenized cow's milk in the presence of an adjuvant, Li et al.⁶⁵ produced a murine model of cow's milk hypersensitivity generated by oral sensitization and challenge resulting in pulmonary reactions. Allergen-specific IgE, histamine release, and intestinal permeability studies combined with histological changes in the lung led the authors to suggest that cow's milk-induced anaphylaxis was the result of Th2-cytokine-mediated mechanisms. Subsequent reports from this group identified T- and B-cell responses to major peanut allergens in the C3H/HeJ model,⁶⁶ as well as strain-dependent induction of peanut allergen by DNA immunization⁶⁷ and ultimately different genetic susceptibility to food allergy linked to different Th1/Th2 responses.⁶⁸ Considering the Th1/T21 phenotype, the combined results suggest that strain, adjuvant, and mode of induction are significant factors that need to be addressed to provide useful mechanisms of human food-induced asthma. Adel-Patient et al.⁶⁹ optimized BALB/c mice to β -lactoglobulin (BLG) to investigate human parameters of asthma including (1) allergen-specific IgE, (2) histamine, leukotriene, and prostaglandin release within minutes after challenge, and (3) bronchial hyperreactivity (BHR), Th2 cytokines (IL-4 and IL-5) in bronchioalveolar (BAL) fluids, and eosinophil infiltration at 24 and 72 hours post challenge. In this model, the investigators found that native BLG, containing conformational epitopes, triggered the release of histamine, LTB₄, LTC₄, and LTE₄, whereas denatured BLG, containing linear epitopes, led to release of histamine and prostaglandin D₂, with virtually no leukotrienes. This dichotomy in mediator release was interesting in that patients with persistent allergy showed higher titers of IgE specific to linear epitopes from alpha- and beta-casein as compared to those achieving tolerance.⁷⁰ Combined results of the above two investigations suggest that a differential role for linear and conformational epitopes may exist in both mice and humans. Continued investigations are needed to address allergen structure and its role in determining allergenicity of proteins.

GASTROINTESTINAL ALLERGY

The gastrointestinal-associated immune system is the largest mucosal system that must differentiate between foreign, self, and nutritive components in the body. The system must be able to balance tolerance versus hypersensitivity based upon foreignness recognition in a Th1/Th2 cytokine environment. The intestinal epithelium provides a barrier, with immunoglobulin exclusion (sIgA, sIgM) and mucus production helping to keep macromolecules from reaching the immune system. Factors that can affect the epithelial barrier and innate immune system include genetics, age, breastfeeding, indigenous microflora, and diet (concentration, nutrient interaction, duration of exposure, and time of exposure). Animal models may not be able to completely extrapolate to human

gastrointestinal allergy — anatomical/physiological/immunological differences, dietary intake, natural diseases, and tools to assess immune responses are but a few obstacles to overcome; however, identifying the proper model and questions addressed to the model will provide significant information relevant to human allergic conditions.

Inbred strains of mice have been characterized as being either high or low IgE-responder animals for both inhalant and food allergens. As in humans, two separate events are required for type 1 immediate hypersensitivity: the first event is a sensitization phase and in the case of mice, the production of two anaphylactic antibodies, IgE and IgG1; the second phase is characterized as the allergic challenge following reexposure to the allergen at the site of the response. In both inhalant and ingestant sensitizations, short-term daily exposures over a period of days or long-term exposure once a week over several weeks have been shown to induce allergen specific IgE/IgG1.

Studies designed in germ-free animals are contributing to delineation of the mechanisms concerning how the immune system is able to deal with the massive antigen challenge represented by commensal and pathogenic flora.^{71,72} These experimental models provide significant insight into the mechanisms maintaining intestinal homeostasis between the diet and bacteria that contribute to inflammation in the intestinal tract. Although little is known about this interrelationship, alteration of the intestinal flora in allergy-prone newborns by feeding them a probiotic (*Lactobacillus*) greatly reduced their development of atopy in the first 2 years of life.⁷³

Dearman et al.⁷⁴ investigated the characteristics that confer allergenic activity on proteins by comparing the immune responses induced in BALB/c mice by bovine serum albumin (BSA) (limited potential to induce sensitization) and OVA (a common food allergen). Groups of mice received protein intranasally or intraperitoneally with or without carrageenan as adjuvant. Irrespective of the route of administration, OVA displayed a significantly greater potential to elicit IgE antibody production than BSA did. Both OVA and BSA stimulated strong IgG and IgG1 antibody responses that clearly demarked the immunogenicity and allergenicity of the two proteins in BALB/c mice. The reciprocal ability of these two allergens to elicit IgE or IgG2a antibody production led the authors to suggest that the integrity of the immune responses is regulated by CD4 Th cell cytokines, which are clearly distinct in mice. Drawing all elements together, the authors proposed that variable responses to OVA and BSA were secondary to the development of divergent T-cell responses, which determines the antibody isotype production observed. The assumption was that the proteins had inherent physicochemical differences that resulted in different protein uptake, processing, and presentation by the immune system.

BN rats and three strains of mice (BALB/c, B10A, ASK) administered OVA by gavage in the absence of adjuvant followed by intraperitoneal challenge were used to assess active systemic anaphylaxis (ASA).⁷⁵ The route of administration (oral sensitization), age of animal (20 weeks), and dose (0.1 mg OVA) had profound effects on sensitization in rats and mice. Serum histamine release and serum IgE levels correlated best in the BN rat and B10A models, suggesting that these animal models are suitable for investigations of IgE-mediated responses to OVA.

With respect to the controversy over maternal sensitization versus tolerance to food antigens, Melkild et al.⁷⁶ identified a polarization of IgE and IgG1 in offspring of mice from ovalbumin-sensitized dams. Depending upon dose, allergen, and exposure, the results suggest that maternal sensitization in mice may lead to IgE tolerance depending upon cytokine balances in the offspring. Contributing to the study of maternal effects on offspring, Lange et al.⁷⁷ showed that maternally transferred monoclonal antibody followed by prophylactic exposure to allergen may be effective in prevention of allergy in humans if their animal model results can be extrapolated to humans. Much research needs to be done on both the maternal sensitization and offspring responses to food and aeroallergens; however, murine models have the distinct advantage of powerful immunological tools to assess the genetics and Th1/Th2 balance in allergic diseases.

The frequency of food allergy identified by single ingredient provocation testing in 25 dogs with histories and cutaneous signs was consistent with food-induced allergic dermatitis.⁷⁸ Although no IgE was measured, elimination diets and provocation tests confirmed that ingredients in soy,

chicken, milk, corn, wheat, and egg provoked positive reactions. Ermel et al.⁷⁹ reported the use of a colony of spaniel/basenji-type dogs that they selected with a genetic predisposition to allergy and histories of sensitivity to pollens and foods. Newborn pups were subcutaneously injected into the axilla with 1 µg cow's milk, beef, ragweed or wheat extracts in alum. At ages 3, 7, and 11 weeks, all pups were vaccinated with attenuated distemper–hepatitis vaccine. At 2 and 9 days after each vaccination, each animal received the same allergen it received as a newborn. Immunized pups responded with allergen-specific IgE by week 3, which peaked at week 26 and could be maintained with bimonthly injections of specific antigen in alum and daily feeding of a maintenance diet containing minimal amounts of the allergen source. Skin tests were consistently positive when challenged with the relevant allergen and negative with an irrelevant allergen (nonimmunized allergen). Endoscopic analysis of gastric food sensitivity testing and histologic tissue examination showed pronounced mucosal swelling and persistent erythema at the site of injection. In addition, late biopsy specimens revealed evidence of eosinophil infiltration into the lamina propria and migration through the endothelium.

This protocol in atopic dogs mimics the following proposed mechanism involved in the development of food allergies in children. In genetically predisposed individuals with an initial early infection or viral challenge, the immune system could be capable of responding to “bystander” antigens with more vigor. The inflamed gut becomes more permeable to proteins, thereby exposing the local immune system to more antigen sources. The symptoms and production of allergen-specific IgE suggest that the inbred allergic dog model could be useful for the characterization of the underlying mechanisms involved in the development of food allergy. Physical symptoms, serum IgE as evidenced by passive cutaneous anaphylaxis (PCA) skin testing, and histologic assessment of the small intestine following oral challenge in peanut-sensitized piglets in the allergic phase showed similar pathologic morphology of the villi, edema of the submucosal small intestine tissue, and vascular congestion as demonstrated in the atopic dog model.⁸⁰ Both large-animal models suffer from a lack of immunochemical tools to assess allergic mechanisms. Continued interest in the area of large-animal models for the characterization of allergic responses and sensitization protocols will provide the immunological tools necessary for detailed mechanistic studies to be performed.

Infant/Hypoallergenic Formulas

Animal studies are being used to determine the effect of potential additives to infant formulas that will replace deficient growth factors and immune stimulators found in maternal milk. Nagafuchi et al.⁸¹ demonstrated that dietary nucleotides upregulated antigen-specific Th1 immune responses in association with an increase in IL-12 production in weanling BALB/c mice. The oral administration of 0.4% dietary nucleotides (proportions similar to human milk at 12 weeks of lactation) in ovalbumine-T cell receptor transgenic (OVA-TCR Tg) mice beginning at 3 weeks of age was regarded as helpful and suggested extrapolation to prevention of human allergies during early infancy in infants not receiving breast milk. Oral administration of a whey protein extract to naturally suckling rat pups downregulated immune activation of spleen cells to orally administered ovalbumin.⁸² The downregulation required prior exposure to oral antigen and was not seen after peripheral antigen sensitization.

The sensitivity and tolerogenicity of infant formulas has routinely been evaluated by determining molecular size and digestibility and using animal models. In a brief communication, Fritsche⁸³ addressed the use of animal models for the evaluation of both allergenicity and tolerizing activity, which can be summarized as follows:

1. Rat peritoneal models appear to be adequate for measuring both the IgE-specific sensitizing and triggering capacities of food allergens.
2. Mouse and guinea pig models are closer to the physiologic sensitization of food proteins.
3. Route of administration, adjuvant use, and reaginic antibody responses should be taken into account, as no ideal animal model exists for food allergy.

Risk Assessment

Beginning in 1996 with the advent of bioengineered food crops, decision trees to assess the potential allergenicity of novel (biotechnology-derived) proteins were being developed. Reports from conferences and workshops are now available to interested individuals. The results of a 2000 National Center for Food Safety and Technology/International Life Sciences Institute/Health and Environmental Sciences Institute (ILSI/HESI) symposium in Summit, Illinois entitled “Genetically Engineered Foods: Assessing Potential Allergenicity” have been published.^{84–87} A workshop organized in 2001 by the National Institute for Environmental Health Sciences (NIEHS), in association with the Environmental Protection Agency (EPA), and the Food and Drug Administration (FDA) that presented various designs and evaluations of animal models to determine the allergenic potential of genetically modified foods in the rat (Penninks and Knippels), mouse (Kimber and Dearman), dog (Buchanan; Hammerberg and Jackson) and swine (Helm) has also been summarized.⁸⁸ Discussions at the 2002 Society of Toxicology meeting held to address progress for assessing strategies, including animal models, for potential allergenicity were summarized by Ladics et al.⁸⁹ The reader is alerted to reports on animal models to detect allergenicity to food and genetically modified products.^{90–92}

A murine model used to distinguish immunogenic properties (route of administration, IgG and IgE production) of novel proteins could distinguish allergenic responses (IgE) versus antigenic (IgG) production.⁹³ Despite confirmed immunogenicity (IgG production) and allergenicity (IgE production) of the three proteins (peanut agglutinin, OVA, and potato acid phosphatase [PPE]) by parenteral injection, BN rats failed to mount an IgE response to the proteins after gavage exposure. Gavage exposure in BALB/c mice elicited both IgG and IgE antibody responses to proteins in a ranking order of known allergenic potency; however, a much more robust response was provoked by intraperitoneal administration of the same proteins.⁹⁴ A differential ability of the rapidly degraded protein, PPE, compared to a more stable protein, OVA, could explain why orally ingested labile proteins fail to invoke antibody responses while those that are more stable to simulated gastric fluid in the stomach may reach mucosal immune systems in the small intestine.⁹⁵ Although PPE was rapidly degraded in simulated gastric fluid (SGF), this protein was able to elicit a substantial IgG antibody response when administered orally. This evidence suggests that the association between stability in SGF and antigenic/allergenic potential may not be solely a function of survivability of proteins in the gastrointestinal tract and that additional factors such as regional enzymatic processing by antigen-processing cells are critical to allergic sensitization.

The relative safety of genetically modified and nongenetically modified soybeans was investigated based upon the annual consumption of soybeans as a food in Korea.⁹⁶ Sprague–Dawley rats were exposed to either a low dose (2 mg/kg) or a high dose (20 mg/kg) of genetically modified soybean versus nongenetically modified soybean homogenates. Passive cutaneous tests and histamine release from rat mast cells receiving either of the soybean homogenates were similar, confirming earlier reports that glycoposphate-tolerant soybeans were as safe as conventional soybeans, based upon IgE-binding studies.⁹⁷

Selgrade and coworkers⁹⁸ have investigated two models, one in BALB/c mice and the other in the BN rat, to assess the effects of air pollution on allergic sensitization as part of toxicological risk assessment. The issues addressed involved sensitization to the house dust mite.^{99–101} Assessment of total and antigen-specific IgE in serum and bronchioalveolar lavage fluids, cytokine levels, and bronchoconstriction by whole-body plethysmography demonstrated both immediate and late phase responses. Although environmental contaminants were shown to exacerbate conditions, the mechanisms are still under investigation to develop methods for application to human health risk assessment. In addition, an entomopathogenic fungus, *Metarhizium anisopliae*, licensed for indoor biopesticide control of cockroaches, was tested for allergenicity using two exposure protocols.^{102,103} Investigations revealed that this organism could potentially induce respiratory allergy in humans. Local responses in the lung (eosinophil, lymphocyte, macrophage infiltration), bronchial alveolar

lavage fluid (BALF) IgE, and functional responses were greater in the intratracheal versus the intraperitoneal sensitized group; however, the systemic IgE response was greater in the intraperitoneal group. The results suggest that the protocol sensitization may play a significant role in the determination of allergen potency and respiratory responses to potential inhalant allergens. Although the research clearly describes environmental contaminants that exacerbate the expression of allergic symptoms, the underlying mechanisms are still being assessed.

IMMUNOMODULATORY/THERAPEUTIC MODELS

The type of effector cells generated *in vivo* is determined by antigen concentration, route of delivery, and the presence of inflammatory signals, which influence antigen-presenting cell activation, upregulation of costimulatory molecules, and cytokine production. In a murine model using intranasal administration of Der p I peptide in association with chitosan, Th2 tolerance to inhalant antigen and reduction of airway eosinophilia were reported; these phenomena may be useful in shifting an effector phenotype from a Th2/Th0 to a Th1/Th0 cytokine response.¹⁰⁴ Peptide alone was insufficient to prevent the induction of Th2-mediated airway inflammatory responses, but peptides adsorbed to chitosan — a compound that enhances uptake of antigen across airway mucosa — was effective in the induction of sustained Th2 tolerance. The feeding of ovalbumin (OVA) dissolved in cow's milk to infant beagles for 28 days proved sufficient to prevent allergy and asthma in adult life as evidenced by airway challenge even with OVA-specific IgE and IgG production as determined by subcutaneous challenge.¹⁰⁵ The mechanism was suggested to be a Th1-protective event of IL-10 and TGF-beta-initiated oral tolerance. In related studies using subcutaneous sensitization of OVA, peanut extract, and recombinant birch pollen allergen, a comparison of contact with allergen on the first day of life versus sensitization at 4 months showed that contact with allergen in early life was decisive for sustained IgE levels and allergic responses.^{106,107} The results suggest that a shift or predominance of Th1 in the Th1/Th2 balance may be evident in the production of tolerance and mucosal system oriented relative to sensitization.

Vohr et al.¹⁰⁸ induced immune reactions in brown Norway rats by dermal and respiratory routes to determine whether draining lymph nodes could be used as early indicators of respiratory sensitization and whether the specificity of the immune-competent cells was the same irrespective of the route of induction. More activated T and B cells were found in the lung-draining lymph nodes after topical induction than after pulmonary induction. Local and distant trafficking of lymphocytes and the doses used to induce the allergic response still need to be investigated; however, this model may be relevant to chemical-induced respiratory hypersensitivity. Coexpression of Th1 and Th2 cytokines during contact allergy was shown to be an important feature of murine contact allergy in BALB/c mice; chemicals differed in the degree of induction of expression of these cytokines but did not induce them in a mutually exclusive manner.¹⁰⁹ In contrast to Dearman and co-workers^{110–113} postulation that divergent cytokine patterns obtained with a variety of contact allergenic chemicals distinguish skin sensitizers from respiratory sensitizers, contact allergic responses did not adhere strictly to the Th1/Th2 paradigm. A significant amount of investigatory work is needed regarding the time-point of application, strain differences, and cells for analysis before predictive tests can be established to predict skin sensitizers and their role in inhalative respiratory allergy induced by chemicals.

Determination of receptor-mediated events in murine models provides provisional information regarding allergic asthma and eosinophilic recruitment. Kaneko et al.¹¹⁴ showed that interaction of antigen with IgE antibody was insufficient in provoking eosinophilic inflammation in BALB/c mice. Inhibition of the IL-4/IL-13 receptor system in BALB/c mice prevented the development of the allergic phenotype; however, both cytokines were regarded as playing minor roles in established allergy.¹¹⁵ Two phenotypically similar models of experimental asthma induced by independent regimens were used to determine transcript profiles associated with each model.¹¹⁶ The results

demonstrated that there was an overexpression of genes encoding molecules involved in the uptake and metabolism of basic amino acids (arginine). Diverse allergens, IL-4, and IL-13 regulated arginase levels in asthmatic lungs of these animals, and arginase levels were shown to be active in human asthma. These controversial findings compared to dogma pertaining to receptor-mediated allergic responses suggest that allergen, dose, strain, and allergy phenotype will need to be carefully evaluated for extrapolation to human allergy.

Using DNA plasmids, the Th1/Th2 bias of the resulting immune response after genetic immunization was shown to be influenced by the nature of the allergen protein, mouse strain, relative ratio of ISS to recombinant protein expressed, and the method of inoculation.¹¹⁷ Oral allergen-gene immunization with chitosan-DNA nanoparticles was shown to be effective in modulating murine anaphylactic responses.¹¹⁸ Slow release and mucosal uptake of particles may be relevant to this method of prophylaxis. Data generated in PLA2 CBA/J mice suggest that allergen-derived long overlapping peptides are able to induce T-cell anergy, decrease allergen-specific IgE, and shift the Th1/Th2 cytokine balance.¹¹⁹ The design of Th1-skewing peptide analogues tested *in vitro* demonstrated modulation of allergen-specific Th2 responses, which was clearly shown to be efficacious in the *in vivo* correlation of Th1- or Th2-skewing characteristics in OVA323-339 TCR transgenic DO11.10 and BALB/c mice.¹²⁰ Sato's group has shown that oral administration of *Blomia tropicalis* or *Dermatophagoides pteronyssinus* can downregulate IgE antibody responses in sensitized mice in a distinct manner and further suggested that anti-IgE could be one mechanism by which protection may be conferred.^{121,122} These experiments may prove helpful in assessing the identification of peptide motifs recognized by human IgG autoantibody antibodies and in the mechanisms underlying anti-IgE therapy under current clinical trials.^{123,124} Additional experiments on the characterization of allergens and peptides, allergen source material, the influence of strain differences, and inoculation parameters will need to be clarified for the various immunotherapeutic clinical responses for the treatment of allergy.

As there is no proven therapy to treat or prevent food allergy, a number of investigations are being undertaken in animal models. In a mouse model of peanut anaphylaxis, mouse recombinant IL-12 was used prophylactically (administered at the time of peanut sensitization) or therapeutically (3 weeks following sensitization) for treatment of peanut allergy.¹²⁵ Both regimens were able to reduce anaphylactic symptoms with marked reductions in plasma histamine levels compared to nontreated controls. Prophylactic treatment with rIL-12 completely blocked peanut-specific IgE production and reduced levels of anti-peanut IgE in therapeutically treated mice. A protective immune response (increased levels of INF- γ and switch to a Th1-type antibody response) was also identified. Although the IgG1/IgG2a ratio was reduced in treated groups, the results were due to a marked reduction in IgG1 rather than an increase in IgG2a. The role of peanut-specific sIgA is also discussed with speculation concerning this antibody's protective effect in the peanut anaphylaxis model. Similar reduced symptomatic scores, IgE levels, suppression of peanut-specific T-cell proliferation, and Th2 cytokine production (IL-4, IL-5 and IL-13) were evident with a therapeutic herbal formula treatment by intragastric gavage.¹²⁶

DNA immunizations have received considerable interest in treating and preventing food allergy in animal models. A primary Th1-type immune response was shown in mice treated with β -lactoglobulin-encoding plasmid (pBLG) compared to control animals receiving BLG or control plasmid.¹²⁷ The efficiency of the preventative effect was highly time dependent, requiring a single intramuscular administration and a latent period of 21 days, leading to inhibition of IgE and an increase in specific IgG2a to BLG. Alum was used as an adjuvant for presentation of the BLG in its native structure, and an association of the immunostimulatory sequence of the plasmid DNA was proposed as a likely reason for the Th1 orientation of the immune response.

Oral administration of a whey protein extract was shown to downregulate immune activation (spleen cell proliferation with TGF- β production and reduced MHC1 expression) to orally administered ovalbumin in suckling rats.¹²⁸ The authors speculated that growth factors present in whey milk could be a potential benefit for formula-fed infants in reducing susceptibility to inappropriate

activation to food allergens. In a guinea pig model of intestinal anaphylaxis, allergic sensitization suggested that the allergenicity of milk may vary according to the proportion of constitutive proteins.¹²⁹ The authors suggested that β -lactoglobulin, which is resistant to gastric pepsin hydrolysis, might be facilitated in the absence of α -caseins, as caseins and β -lactoglobulins are tightly linked into casein micelles.

With the inverse relationship between atopy and infectious disease, species of *Lactobacillus* have received considerable interest as a probiotic therapy in preventing food allergy. Shida et al.¹³⁰ developed a novel IgE response model using ovalbumin T-cell-receptor transgenic (OVA-TCR-Tg) mice to investigate the regulatory role of heat-killed *Lactobacillus* (LcS) originally isolated from humans. Intraperitoneal administration of LcS during the OVA oral sensitization period led to increased IL-12 production and inhibited both IgG1 and IgE production, resulting in reduced systemic anaphylaxis.

Although the mechanisms of probiotic therapy are yet to be determined, lactic acid bacteria may provide a beneficial antiallergic effect in the treatment of allergy.¹³¹ Heat-killed lactic acid bacteria administered to TCR transgenic mice suppressed both IgE and IgG1 responses, suggesting this strong IL-12 potentiator may prevent food allergy. Lee et al.¹³² demonstrated that oral administration of IL-12 3 weeks after peanut sensitization attenuated anaphylactic reactions triggered by peanut challenge in allergic mice. Using the murine peanut allergy model, Li et al.¹³³ were able to demonstrate that immunotherapy with modified peanut proteins and heat-killed *Listeria monocytogenes* was effective in treating peanut allergy. These reports suggest that cytokine balance, dose, and timing of either interleukin therapy or probiotic treatment may be useful in treatment of certain human allergic diseases. Although controversial, not having an identified mechanism for blocking peanut-induced anaphylaxis in a murine model, Li et al.¹²⁶ suggested that traditional Chinese medicines/treatments may be useful in preventing anaphylactic episodes in peanut-sensitized mice. A food allergy herbal formula — active contents unknown — both protected against anaphylaxis and reversed an ongoing IgE-mediated peanut response in C3H/HeJ mice. Alternate immunotherapy regimens continue to be investigated; however, more mechanistic studies and clinical trials need to be performed before they can be accepted as alternate sources of therapy. These investigations may contribute to understanding of the antiallergic mechanisms of lactic acid bacteria in the treatment of food allergy.

CONCLUSION

Challenges in animal model selection include:

1. Identifying natural allergy models
2. Interspecies extrapolations
3. Imperfect or biased knowledge; informed choices
4. Pure or enriched novel proteins versus presence in diet
5. Anatomical, physiological, and immunological differences
6. Route, dose, and timing of allergen administration
7. The use of adjuvants

Each of these circumstances must be taken into account when data are to be extrapolated to human allergic diseases.

Murine animal models predominate in the literature with regard to the allergy-related topics reviewed in this article. Their prominence is principally related to the better-characterized immunology, ease of handling, and lower cost to the investigator, permitting a greater degree of flexibility in protocol design. Strain difference, route of administration, and expected clinical symptoms are still problematic and are often not reproducible, depending upon experimental designs and the food

source or allergen. Results from high-IgE-producing dogs and the similarity of natural food-allergic immune responses in both swine and canines to humans suggests that these higher animal species could be used to assess immunoregulatory mechanisms and the relative allergenicity of novel proteins. Animal models are being increasingly used to determine mechanisms to induce tolerance by way of prophylactic treatments as well as intervention or therapeutic options to reduce food allergy. In each report, significant information is made available that contributes to our knowledge of the immunopathogenesis of human allergy.

In summary, several animal models, including mice, rats, dogs, and swine, are currently being investigated for their value in the mechanisms, therapy, and prediction of allergenicity of food, plant, and animal substances that may be extrapolated to different human allergic traits. To date, no animal model is predictive, and the research designs differ significantly in route of administration, adjuvant use, dose, strain, and allergen versus nonallergen profiles, requiring additional standardization to be of significant value in predicting allergenicity of novel proteins. Allergenic sensitivity may be determined not by whether a novel protein is capable of sensitizing but by its concentration. Questions remain regarding the margin of safety at which the risk/benefit ratio is acceptable and include:

What criteria should be applied, e.g., what is the threshold dose for sensitization/allergic responses?

What are the dose effects with potency profiling?

What population should risk be directed toward — sensitized or sensitive?

What is the health risk — frequency of exposure, amount of exposure, and intensity of the allergic reaction?

Finally, it is the responsibility of investigators to use “proof of concept” when addressing experimental design in animal models to impart information related to mechanism, therapy, and predictive protocols that will have an impact upon human allergic diseases by identifying health risks, e.g., frequency of exposure, amount of exposure, and intensity of reaction.

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CHAPTER 8

Animal Models for the Study of *Helicobacter* Infection

Akira Nishizono and Toshio Fujioka

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INTRODUCTION

Helicobacter Species and Diseases

The genus *Helicobacter* comprises a group of microaerophilic Gram-negative spiral to curved bacteria isolated from the stomachs, intestines, and hepatobiliary tracts of humans and animals.¹⁻⁶ The type species of the genus, *Helicobacter pylori*, has been recognized in humans as the most common cause of gastroduodenal diseases.⁷ *Helicobacter pylori* infections occur in human populations throughout the world, and the prevalence of infection increases with age. Infection occurs at an early age (i.e., childhood) and is more prevalent among individuals in developing countries than among those in developed countries.⁸ More than half the individuals in developed (middle to old age) and developing (all age) countries are infected in adulthood, and *H. pylori* infection is one of the most common bacterial infections in humans. Once acquired, the infection becomes chronic and persists for life unless treated, but most individuals infected with *H. pylori*

remain asymptomatic for decades.⁹ Although the primary route of *H. pylori* infection remains poorly understood, it is suggested that fecal–oral or oral–oral transmission may occur via water, food, or the nursing process.^{10,11} *Helicobacter pylori* infection is a significant risk factor for peptic ulcer disease (gastric and duodenal ulcer), and moreover, chronic atrophic gastritis and intestinal metaplasia following chronic superficial gastritis are risk factors for adenocarcinoma of the stomach, since the prolonged inflammation accompanying *H. pylori* infection might contribute to the development of this malignancy.^{12,13} In addition, this bacterium may play a role in the development of low-grade B-cell lymphomas of gastric mucosa-associated lymphoid tissue (MALT lymphoma).¹⁴

Although the exact mechanism by which *H. pylori* produces gastric inflammation has not been established, a number of hypotheses have been proposed. Two major factors may account for the gastric damage. One is a bacterial factor contributing to *H. pylori*–associated disease. Most *H. pylori* isolates, as well as each of the gastric *Helicobacter* spp., produce large quantities of the enzyme urease.¹⁵ This enzyme plays a role in the initiation of infection or in permitting survival of the organism in the gastric mucosa.¹⁶ By generating ammonia, urease might play an important role in mediating bacterial survival in acidic conditions, and ammonia might be cytotoxic to gastric epithelial cells and enhance neutrophil-dependent gastric mucosal cell injury.¹⁷ Moreover, at a genetic level, *H. pylori* strains are highly diverse, but it appears that all strains have essentially an equal capacity for long-term colonization of the human stomach, despite such differences. The most significant diversity thus far identified in *H. pylori* is the presence of the *cag* pathogenicity island (*cag* PAI).^{18,19} This region of approximately 40 kb encodes about 25 genes, including several that are homologs of secretion genes in other bacteria.^{20,21} In western populations *cag*+ strains have been shown to be more virulent than *cag*– strains, with an increased lifetime risk of developing adenocarcinoma of the stomach associated with the former.¹⁸ Colonization with *cag*+ strains is associated with more intense gastric inflammation, increased risk of development of atrophic gastritis, and increased production of proinflammatory cytokines (such as IL-8).^{22–24} More recently, *cag*+ strains have been shown to translocate the bacterial protein CagA into gastric epithelial cells by a type IV secretion system. CagA is tyrosine-phosphorylated and induces changes in the intracellular signal transduction process.^{25–27} Another marker of diversity is the ability of *H. pylori* strains to induce vacuoles in cultured cells.²⁸ Unlike the situation with the *cag* gene, all *H. pylori* strains possess the gene encoding cytotoxin (VacA).²⁹ However, *vacA* exhibits genetic polymorphism, and a correlation has been found between *vacA* genotypes and the gastroduodenal disease associated with the *H. pylori* infection in individual patients.³⁰

The other important factor associated with gastric lesions is innate and acquired host immune responses and subsequent disease development. Individuals infected with *H. pylori* have consistently induced systemic and local humoral immune responses against the organism.^{31,32} Perhaps *H. pylori* persists in the gastric environment despite the strong humoral host response because the organism is protected within the gastric mucus. In the initial stages of *H. pylori* infection, *cag*+ strains activate host cell signal transduction, including induction of the transcription factor NF- κ B, with consequent secretion of the proinflammatory cytokine IL-8.²² Following such acute inflammatory reactions, immune responses associated with helper T cells are induced. Cellular immune responses to *H. pylori* have been studied for humans and mice, but the nature of such responses, including the possible involvement of protective immunity, is not well defined. Th1 cell-dominant immune responses occur in the stomach, as indicated by the marked expression of IFN- γ mRNA.³³ In comparison, a dominant Th2 response induces minimal inflammation and may progress to a severe B-cell lymphoproliferation, as seen in Balb/c mice. Such cytokine induction or imbalance (Th1/Th2) by *H. pylori* indicates that extensive signaling occurs between gastric epithelial cells, lymphocytes, and antigen presenting cells, macrophages, or dendritic cells.³⁴ Intriguingly, synergistic infection of C57BL/6 mice with *Helicobacter felis* and a helminth, *Heligmosomoides polygyrus*, which elicits a polarized Th2-shifted response, reduced the severity of gastric inflammation.³⁵

Purposes and Choice of the Animal Model

A potential role of *Helicobacter pylori* in the pathogenesis of peptic ulcer disease was recognized soon after the bacterium was discovered. Ingestion of *H. pylori* by human volunteers results in gastritis, and eradication of *H. pylori* infection resolves symptomatic gastritis and peptic ulcer diseases.¹ However, in the initial stages of *Helicobacter* research, progress in understanding the pathogenesis of *H. pylori* infection was limited by the lack of a suitable experimental animal model and the restricted host range of *H. pylori*.^{36,37} According to the classical Koch's postulate, the etiological organism will reproduce the disease when administered to a "susceptible experimental animal."³⁸ Many investigators have attempted to adapt several animals as experimental animal models for *H. pylori* infection. First, gastritis similar to that found in humans was established by several investigators in germ-free mice, gnotobiotic piglets, and gnotobiotic beagle dogs.^{36,37,39} Germ-free or microbe-defined (gnotobiotic) conditions provide the opportunity to study the "pure" infection process without the complicating factors of intercurrent subclinical infections and the confounding effects of commensal microbiota and their products. In fact, the chronic *H. pylori* infection state seems to be established with the aid of numerous host factors (gastric physiology such as acid output, bloodstream, amount of mucin, innate and acquired immune responses, commensal microflora in the intestine). There is therefore a need to develop a more convenient and natural animal model for the study of *H. pylori* infection. In particular, experimental animal models of *H. pylori* infection are indispensable in clarifying the pathogenic significance of this bacterium in gastric malignancies. The International Agency for Research on Cancer (IARC) examined the evidence linking a number of infectious agents with human cancer.⁴⁰ Although evidence in experimental animals for carcinogenicity of infection with *H. pylori* is inadequate, it was designated as a definite carcinogen (Group I) to the human stomach based on the prospective case-control studies.^{12,13,41} This chapter presents an overview of animal models for the study of *Helicobacter* infection and describes the characteristics of a representative animal model for the study of *H. pylori* infection (Table 8.1).

ANIMAL MODELS OF *H. PYLORI* INFECTION

Nonhuman Primate Models

The use of nonhuman primate animals for the study of *H. pylori* infection is useful methodology because anatomical, physiological, and dietary features of stomachs in nonhuman primates are similar to those of humans. The 10- to 20-year lifespan of nonhuman primates facilitates long-term follow up with endoscopy and repeated histological examinations of gastric mucosa using biopsy specimens. There have been several successful reports of the experimental transmission of *H. pylori* in chimpanzees (*Pan troglodytes*)⁴² and species of macaques: rhesus monkey (*Macaca mulatta*),⁴³ cynomolgus monkey (*Macaca fascicularis*),⁴³ and Japanese monkey (*Macaca fuscata*).⁴⁴ Although chimpanzees have not been widely available for *H. pylori* studies in recent times due to community concerns about their use, some kinds of *Macaca* species are available for a wide variety of research purposes. Several reports of experimental *H. pylori* infection using rhesus monkeys have been published.⁴³ We have developed the Japanese monkey model of *H. pylori* infection, and it also appears to be a useful and promising nonhuman primate model.⁴⁴

Wild Japanese monkeys live all over the Japanese islands, and their habitat is similar to that of the Japanese human population. Japanese monkeys appear to be suitable as a nonhuman primate model for human *H. pylori* infection for the purpose of clarifying the pathogenesis of *H. pylori* in gastric and duodenal lesions. Prior to experimental infection, endemic spiral bacilli (*Helicobacter heilmannii*-like organism: HHLO, former name *Gastrosprirum hominis*) are naturally colonized in the stomach of most wild (not colony-bred) monkeys. HHLO is generally considered to be a viable

Table 8.1 Animal Models for the Analysis of *Helicobacter* Infection

Animal	Species of <i>Helicobacter</i>	Advantages	Disadvantages
Nonhuman primates	<i>H. pylori</i>	Gastric physiology similar to human; possible long-term follow up with repeated endoscopy	Expensive; difficult to breed; naturally colonized by HHLO; lack of inbred host
Gnotobiotic piglets	<i>H. pylori</i>	Gastric physiology similar to human; easy to study the effect of microflora	Expensive and difficult to breed
Ferrets	<i>H. mustelae</i>	Natural colonization; gastric physiology similar to human	Different colonization pattern from human; chronic gastritis only
Mice (inbred)	<i>H. pylori</i> (SS1) <i>H. felis</i>	Most available and economical; good for testing vaccines or antibiotics; availability of transgenic or knockout animals; easy to study the effect of host immune status	Different findings from human gastric lesions; no ulcer and cancer
Mongolian gerbils	<i>H. pylori</i>	Possible to induce severe acute/chronic gastritis, ulcer, intestinal metaplasia, atrophy; mimic to serial gastric lesions in human; adenocarcinoma induced by infection alone or in combination with carcinogen	Different findings from human gastric lesions; lack of immunological backgrounds and reagents; unavailability of transgenic or knockout animals
Rats	<i>H. felis</i>	Similar to human gastritis	Lacks polymorphonuclear cell infiltration
Gnotobiotic dogs	<i>H. pylori</i>	Gastric physiology similar to human	Expensive and difficult to breed in SPF conditions
Cats	<i>H. felis</i>	Gastric physiology similar to human	Colonized by HHLO (domestic cats)

but nonculturable (VNC) bacterium. Gastric injury induced by this organism is not apparent, even though numerous bacteria colonize the gastric mucosa of monkeys and this phenomenon is also observed in HHLO-infected gastric mucosa of human cases⁴⁵ (Figure 8.1). Similar to other *Helicobacter* spp., HHLO possess strong urease activity, and presumably it is desirable to eradicate this organism prior to experimental infection with *H. pylori*.

For the inoculation experiment with *H. pylori*, the animals were given ampicillin syrup (30 mg/kg body weight orally) for 14 days to eradicate HHLO in the stomach. Sodium bicarbonate (1 g/day orally) and famotidine (20 mg/kg intramuscularly) were given for 3 days prior to challenge infection. The *H. pylori* inoculum used was a broth culture suspension grown under microaerobic conditions and prepared from fresh clinical isolates. Food was withheld from the animals for 48 hours before inoculation, and only tap water was given. Each animal was anesthetized with ketamine hydrochloride, and a 5-ml suspension (10^9 colony forming units [CFU]/ml) of *H. pylori* (a mixture of the four clinical isolates) was sprayed endoscopically around the antrum via a tube. The gastric mucosa was examined endoscopically, and gastric biopsy specimens were collected from the antrum before challenge and at 7, 14, and 28 days, 6 and 12 months, and 2, 3, 4, and 5 years after inoculation, both from infected and uninfected animals.

One week after inoculation, macroscopic gastritis showing with erythema and erosions was noted, and these findings were consistent with the acute gastric mucosal lesion (AGML) observed in human cases. Colonization of *H. pylori* was determined by bacterial culture and the rapid urease test (RUT), using the recovered gastric biopsy specimens. Histologically, spiral bacteria were detected in the infected animals by Giemsa or Warthin–Starry stain (Figure 8.2). Furthermore, the elevation of *H. pylori*-specific IgG in plasma was determined by enzyme-linked immunosorbent assay (ELISA). In the early phase of infection, polymorphonuclear cells and leukocytes dominantly infiltrated the edematous lamina propria. After 3 months, mainly mononuclear cells and plasma cells infiltrated the lamina propria layer. The gastritis scores of the antral mucosa in *H. pylori*-infected Japanese monkeys changed during the 5-year observation period, reaching a maximal score at 1 week after inoculation and then decreasing gradually. A significant difference remained evident between the infected and uninfected animals. Six months after inoculation, the

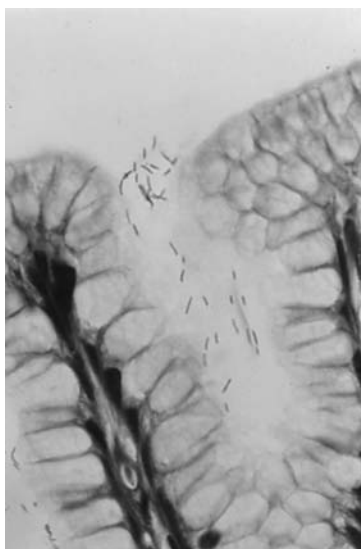


Figure 8.1 Photomicrograph of gastric mucosa of an *H. pylori*-infected Japanese monkey (H&E; original magnification, $\times 1000$). Note the presence of 2 to 5 μm , one to three loose spiral bacteria, which are present in the bottom of the mucus layer adjacent to the superficial epithelial cells and intense mononuclear and polymorphonuclear cells in the lamina propria.

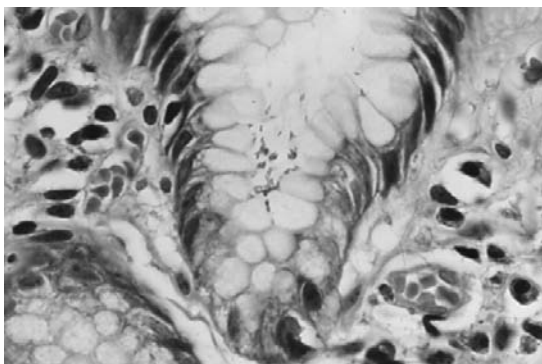


Figure 8.2 Photomicrograph of gastric mucosa of an HHLO-colonized Japanese monkey (Hematoxylin-eosin: H&E; original magnification, $\times 1000$). Note much longer body than *H. pylori* (5 to 10 μm), tight spiral shape, and the absence of inflammatory infiltration in the lamina propria.

pyloric glandular height was apparently lower in the infected animals than in controls, a finding that was sustained throughout the 5-year observation period.⁴⁶ Cell proliferation activity in the antral mucosa of infected animals was significantly accelerated throughout the entire observation period. These consequences demonstrated that *H. pylori* infection in the stomach caused gastric cell damage and acceleration of proliferative cell kinetics, which may explain the potential mechanism for the causal role of *H. pylori* in the chain of events leading to gastric carcinoma.

This monkey model facilitates investigation of the correlation between the long-term sequence of gastric mucosal changes and pathophysiological parameters, such as gastritis score, reduction of glandular height and increase in the number of cells immunopositive for Ki-67 and p53 proteins.⁴⁶ Oda and co-workers demonstrated the rate of p53 gene mutation in Japanese monkeys infected with *H. pylori* human isolates for between 1.5 and 7.5 years. Genetic alterations in exons 5, 6, 7, and 8 of the p53 gene were uncommon in the *H. pylori*-uninfected monkeys, whereas a higher prevalence of missense mutations in the p53 gene appeared in association with *H. pylori* infection, which might be closely associated with the degree of gastric mucosal atrophy, which in turn reflects the duration of *H. pylori* infection in the gastric mucosa.⁴⁷

Nonprimate Models

Mouse Model

Consequent to the revelation that *H. pylori* was closely associated with the development of gastritis and peptic ulcer disease in humans, the need arose for an experimental animal model in which pathogenicity, transmission,⁴⁸ vaccination,^{49,50} and chemotherapeutic intervention could be evaluated. Initially in *Helicobacter* research, the restricted host range of *H. pylori* limited investigation of the pathogenesis of the disease and the only animal models available were the germ-free piglet and nonhuman primates. However, small animals such as mice and rats are particularly well suited as experimental hosts because of their size, ease of manipulation, and genetic backgrounds.

Evidence that gastric colonization with *H. pylori* could occur in mice was first reported by Karita et al.⁵¹ In their work, athymic nude Balb/c mice were challenged with clinical isolates of *H. pylori* and evaluated for infection. No colonization occurred with type strain NCTC 11637, however, all three clinical isolates showed colonization of the fundic and pyloric regions of all nude mice for at least 20 weeks. However, the nude mouse model has some problems for evaluation of real host responses to and pathogenicity of *H. pylori*. Lee et al. isolated *Helicobacter felis*, which colonizes the stomachs of cats. This facilitated the use of small animal models of gastric infection using germ-free and specific pathogen-free (SPF) Swiss Webster mice.^{52,53} Their established strain

of *H. felis* (type strain ATCC 49179: CS strain) colonized the stomach in large numbers in mucus and deep in the gastric pits and induced the same gastric lesion as that found in humans infected with *H. pylori*.^{52,53} At 2 weeks post-infection, an acute inflammatory response was seen, comprising eosinophils and neutrophils. At 3 weeks, the polymorphonuclear response was more prominent, with large numbers of neutrophils, and lymphocytes also increased in number. By week 8, several relatively large lymphoid nodules were present in the submucosa. This bacterium showed progression from acute inflammation to persistent or chronic inflammation as is seen in human infection with *H. pylori*. Since *H. felis* lacks *cag* PAI and *vacA* and recent studies have revealed that the properties of *cag* PAI and *vacA* have high potential to induce gastric lesions, *H. pylori* is a more suitable inoculum for experimental infection in animal models. Marchetti et al. reported that several clinical isolates could successfully colonize the stomach, using SPF conditioned mice (CD1 mice), and could induce gastritis as seen in the human stomach.⁵⁴ Significant colonization was demonstrated for at least 8 weeks in SPF CD1 mice as well as conventional CD1 and Balb/c mice. However, the level of colonization in previous studies was very low (approximately 10^3 CFU/10 mg gastric tissue), and bacteria could not be easily observed by microscopic evaluation.⁵⁴ With such poor colonization and low levels of recovered bacteria, it was difficult to compare this model with human infection. Because of these situations, inbred mice colonized with a *H. pylori* laboratory strain are the most suitable model for the analysis of *H. pylori*-induced diseases.

In 1997, Lee et al. isolated a colony with very good colonizing ability after screening fresh clinical isolates and long-term adaptation in mice.⁵⁵ The Sydney strain of *H. pylori* (strain SS1), is *cagA*- and *vacA*-positive and can achieve a high level of colonization ($10^5 < \text{CFU}/10 \text{ mg}$ gastric tissue) in SPF inbred mice.⁵⁵ The SS1 model provides a reproducible and simple method for ensuring uniform colonization of animals suitable for evaluation of *H. pylori*-associated diseases. The level of colonization varies depending on the mouse strain. High colonization of SS1 in the stomach is noted in C57BL/6 mice, and histological consequences of long-term infection with SS1 are very similar to the histological findings in *H. pylori*-infected human stomach. The development of chronic gastritis during a period of 8 months after infection with SS1 is shown in Figure 8.3. A striking feature is that the inflammation is clearly more severe in the transitional zone between the antrum and body mucosa. Our standard procedures⁴⁹ for this model are as follows. SS1 stock bacteria are stored at -80°C in Brucella broth containing 7% heat-inactivated horse serum and 10% dimethyl sulfoxide (DMSO). After thawing, bacteria are grown on 7% horse or sheep blood agar plates for 4 to 5 days at 37°C , in microaerophilic conditions. Two to three streaks out of the lawn by swab are inoculated into 7 ml of Brucella broth containing 7% heat-inactivated horse serum, and bacteria are cultured overnight with shaking at 37°C in microaerophilic conditions. Usually,

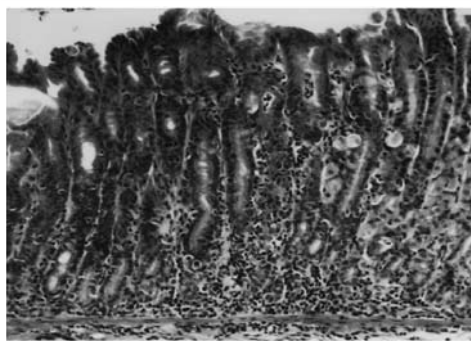


Figure 8.3 Photomicrograph of gastric mucosa of an *H. pylori* SS1-infected C57BL/6 mouse at 18 weeks after infection (H&E; original magnification, $\times 200$). Note severe inflammatory cell infiltration dominated by mononuclear cells, especially in the transitional zone between the antrum and the corpus.

adult mice are inoculated with 1 ml and infant mice with 0.5 ml of broth culture (approximately 10^7 bacteria). After inoculation, food intake is prohibited for 6 hours with free access to water. Serial changes of lesions associated with *H. pylori* in mice were as follows: no apparent histological changes occurred during the first 4 weeks, and then chronic active gastritis developed characterized by infiltration of lymphocytes and plasma cells in the lamina propria. Infiltration of polymorphonuclear cells was not prominent in gastric tissue throughout the whole observation period. Elevation of *H. pylori*-specific antibody occurred about 4 weeks after inoculation as determined by ELISA.⁴⁹

An advantage of using mouse models is the availability of so many transgenic and genetic depletion (knockout) strains for investigation of *H. pylori* infections. This is particularly useful with regard to putative pathogenic factors, such as induction of inflammatory cytokines and immune state, and for the design of a vaccine candidate against this infection. Mohammadi et al. clearly demonstrated that helper T cell cytokines greatly impacted the formation of *H. pylori*-induced lesions.³⁴ The *H. pylori*-dependent cellular response had a Th1 phenotype characterized by local and systemic elevation of IFN- γ and undetectable levels of IL-4 or IL-5 in both challenged mice following vaccination (protected by vaccination), and infected unvaccinated mice. These researchers also demonstrated the role of the IFN- γ -dependent inflammatory response against *H. felis* by *in vivo* neutralization of IFN- γ in splenic and gastric lymphocytes from vaccinated or unvaccinated mice. Moreover, they confirmed that the reduction in *H. felis* colonization was correlated most significantly with an increase in antigen-specific Th2 cytokines using a *H. felis*-infected IL-4 $-/-$ mice model. Nedrud et al. also reported the importance of helper T cell function using knockout mice.⁵⁶ Although humoral immunity, especially secretory IgA, probably played a major role in preventing or eradicating *Helicobacter* infections, these authors demonstrated that T cells may play a role in protection from *Helicobacter* infections in IgA-deficient mice and μ MT B cell-deficient mice.⁵⁷

More recently, Fox et al. reported that *H. pylori* infection induced gastric adenocarcinoma in an experimental mouse model of disease.⁵⁸ They demonstrated this phenomenon using transgenic hypergastrinemic (INS-GAS) mice⁵⁹ infected with *H. pylori* that possesses the cancer-associated *cag* determinant (*cagE*). All male INS-GAS mice infected with *H. pylori* developed atrophy, intestinal metaplasia, and dysplasia by 6 weeks postinoculation, and adenocarcinoma by 24 weeks postinoculation. Inactivation of *cagE* delayed the progression of carcinoma, but malignancy developed eventually in all males infected with *H. pylori*. In contrast, none of the *H. pylori*-infected female mice developed carcinoma, and mucosal injury scores of the stomach in females were significantly lower than those in males. This description parallels the epidemiologic features of intestinal-type gastric carcinoma in humans, and loss of *cagE* temporally retards but does not abrogate disease progression.

Mongolian Gerbil Model

The mongolian gerbil (*Meriones unguiculatus*) is a small rodent similar to the rat; it has been used for the study of epilepsy and parasitic infections. The experimental *H. pylori* infection model using gerbils was initially reported by Yokota et al. During 2 months after inoculation, only a mild inflammatory reaction was observed in the gastric mucosa of the infected animals administered *H. pylori* combined with indomethacin.⁶⁰ Hirayama et al. demonstrated that gastric ulcers and intestinal metaplasia were produced 6 months after inoculation with *H. pylori* in this model.⁶¹ Such pathological changes subsequently confirmed similar sequences that demonstrated that changes in the gastric mucosa were similar to those found in human *H. pylori* gastric mucosal lesions. Although the information about this model (immunological background, cytokine profiles, and tumorigenicity) is still preliminary, the model is an important advance with considerable scope for investigation of the mechanisms of pathogenesis and factors important in the long-term progression from atrophic gastritis to gastric carcinoma.^{62,63}

In our gerbil model we used both a clinical isolate and type strain ATCC 43504 as the *H. pylori* inoculum. Live bacteria (10^9 CFU/ml) grown in broth culture suspension under microaerobic

conditions were orally administered directly to the stomach by solid catheter (4 Fr size). Animals were housed five per cage, and food was withheld for 24 hours before inoculation. The animals were given water *ad libitum* for 12 hours following inoculation. Histopathological examination, bacterial culture, and determination of serum anti-*H. pylori* antibody elevation was performed 1, 2, 3, and 6 months after inoculation. The spiral bacteria were observed in the mucus and gastric pits of all inoculated animals from 1 month after inoculation throughout the whole observation period. However, nearly half of the animals had barely detectable *H. pylori* in the stomach by bacterial culture. The bacterial counts from the stomachs of gerbils 1 and 6 months after *H. pylori* inoculation were 25 and 410 CFU/10 mg of gastric tissue, respectively.⁶⁴ These levels of colonized bacteria were nearly 1/10 to 1/100 those of human and monkey.

Infected animals carrying the spiral bacteria in their stomach throughout the observation period showed various degrees of gastric mucosal damage. Macroscopically, the stomachs obtained from *H. pylori*-infected gerbils had irregularly thickened walls and spotty hemorrhages and erosions (Figure 8.4A). Histopathological changes of gastric mucosa from the early period to the chronic phase after inoculation are described below.⁶⁴ A severe infiltration of the lamina propria by polymorphonuclear and mononuclear cells was observed, and the submucosal layer was infiltrated by mainly mononuclear cells, with formation of lymphoid follicles (Figure 8.4B). Pyloric glands were dilated, and some penetrated the lamina muscularis. These findings were prominent 3 months after inoculation (gastritis cystica profunda) (Figure 8.4C). Gastric ulcers (Figure 8.4D), gastritis cystica profunda, atrophic changes of the mucosal layer (Figure 8.4E), and goblet cell metaplasia (Figure 8.4F) occurred between 3 and 6 months after inoculation.

Studies of Carcinogenicity in H. pylori-Infected Gerbils

Fox et al. demonstrated a possible carcinogenic role for *Helicobacter* species in the gastric mucosa after oral administration of *N*-methyl-*N*-nitro-*N'*-nitrosoguanidine (MNNG) in ferrets infected with *Helicobacter mustelae*.⁶⁵ This was the first study using carcinogens combined with infection with a *Helicobacter* species. However, spontaneous adenocarcinomas have been observed in stomachs of aged ferrets with *H. mustelae* even in the absence of carcinogen administration. In 1998, Tatematsu et al. reported that glandular stomach carcinomas developed in gerbils exposed to nitroso compounds alone.⁶⁶ They confirmed that oral administration of MNNG and *N*-methyl-*N*-nitrosourea (MNU) resulted in the development of adenocarcinoma in the glandular stomach of *H. pylori*-infected gerbils. Sugiyama et al. reported the development of carcinoma in the gastric mucosa of gerbils at 40 weeks after inoculation at 7 weeks of age with *H. pylori* (type strain ATCC 43504); the gerbils were given a range of doses of MNU before or after bacterial inoculation.⁶⁷ The most intriguing result was that only the groups of animals exposed to both *H. pylori* and MNU developed gastric cancers. Interestingly, two different types of adenocarcinoma, intestinal type and diffuse type, developed at significantly higher frequencies.⁶⁷ Tokieda et al. conducted a study in which 5-week-old gerbils were inoculated with *H. pylori* and given MNNG at 50 µg/ml orally for 20 weeks for comparison against animals administered MNNG alone.⁶⁸ At 52 weeks after inoculation, the stomachs were analyzed histopathologically. The group treated with MNNG and *H. pylori* developed gastric carcinoma at a significantly higher frequency than the group treated with MNNG alone. Evaluation of cell proliferation of the pyloric mucosa using a labeling index of 5-bromo-2'-deoxyuridine (BrdU) revealed a marked acceleration of the labeling index in the animals infected with *H. pylori*, irrespective of MNNG administration. This result suggests a synergistic link between *H. pylori* infection and chemical carcinogenic action in gastric carcinogenesis. Such enhancement of gastric carcinogenesis by *H. pylori* infection was also described by Shimizu et al. using a gerbil model treated with MNNG.⁶⁹

As described above, *H. pylori* infection apparently increases the risk of chemical carcinogenic action in the gerbil model, but the most remarkable feature is the direct link between *H. pylori* and carcinogenesis. Watanabe et al. demonstrated that gerbils inoculated with clinical isolates of *H.*

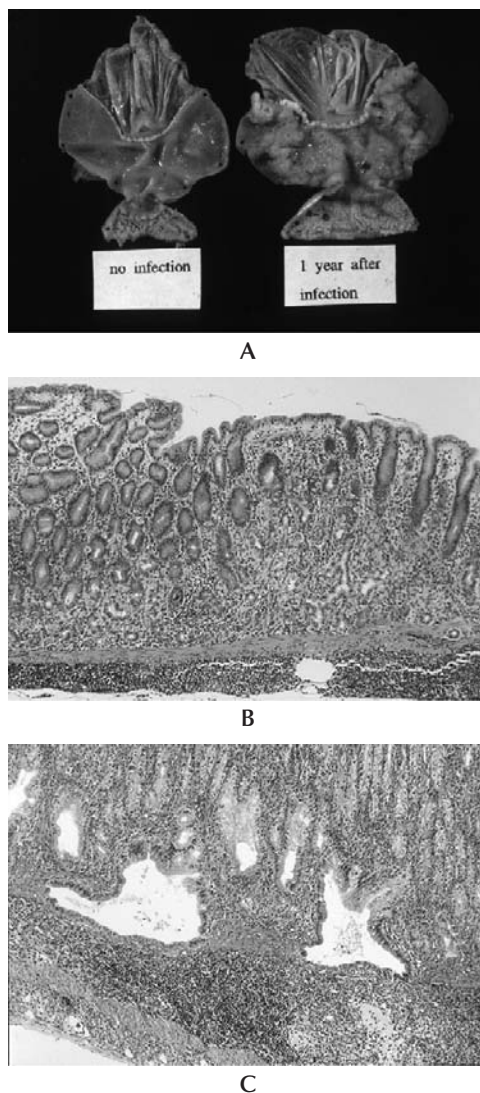


Figure 8.4 Photomicrograph of gastric mucosa of an *H. pylori* 43504 strain–infected gerbil. A: Macroscopic findings of the stomach of uninfected (left) and infected (right) gerbils. B: Photomicrograph of gastric mucosa of an infected gerbil at 18 weeks after infection (H&E; original magnification, $\times 100$). Note thickened gastric wall with severe inflammatory cell infiltration dominated by mononuclear cells. C: Some of the dilated glands destroying the lamina muscularis and extending to the submucosal layer in infected gerbils 6 months after *H. pylori* infection (H&E; original magnification, $\times 100$).

pylori obtained from a patient with gastric ulcers developed well-differentiated adenocarcinoma (10 of 27 gerbils) at 62 weeks after inoculation.⁶³ Honda et al. also reported the occurrence of adenocarcinoma in *H. pylori* (ATCC 43504 type strain)–infected gerbils at 72 weeks after inoculation.⁶² Both studies provided similar findings about sequential histopathological changes. Chronic active gastritis developed initially in the lesser curvature of the antrum, accompanied by infiltration of polymorphonuclear and mononuclear cells, extending eventually to the fundic regions. Intestinal metaplasia then became severe and was associated with dysplasia at 12 and 18 months after *H. pylori* inoculation. At 18 months after the sequential histopathological changes in the gastric mucosa, well-differentiated adenocarcinoma was found in the pyloric regions of the gerbils. All the gastric cancers reported in the two studies developed in the pyloric regions, were of a well-differentiated type, and very closely resembled the histopathological changes in human gastric

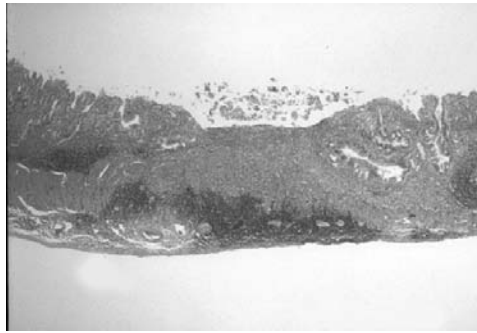
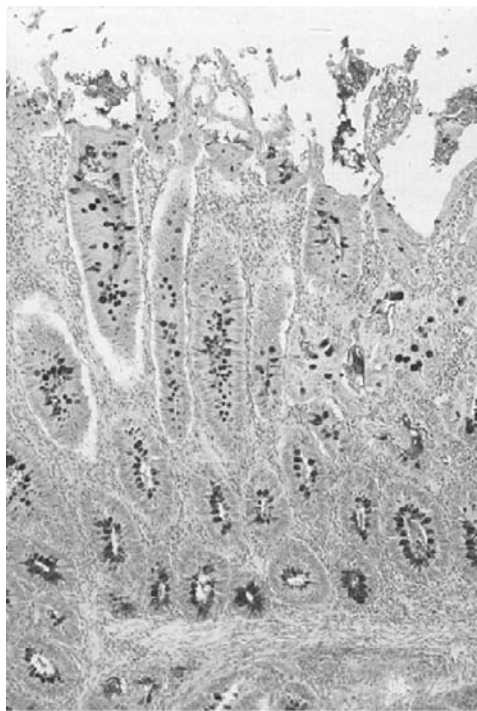
**D****E****F**

Figure 8.4 (continued) Photomicrograph of gastric mucosa of an *H. pylori* 43504 strain–infected gerbil. D: Gastric ulcer was seen in the infected gerbils 6 months after *H. pylori* infection (H&E; original magnification, $\times 40$). E: Mucosal atrophy with a reduced number of pyloric glands and loose interstitial with a small number of inflammatory cells in infected gerbils 6 months after *H. pylori* infection (H&E; original magnification, $\times 100$). F: Note goblet cell metaplasia in the infected gerbil 6 months after *H. pylori* infection (Alcian blue stain; original magnification, $\times 200$).

mucosa caused by *H. pylori* infection. The possible mechanism through which *H. pylori* infection may result in a predisposition to gastric carcinoma is addressed by this model. Ogura et al. examined the roles of *vacA* and *cagE* in the gastric pathogenicity of *H. pylori* using a long-term (62-week) gerbil model.⁷⁰ Both genes are associated with the virulence of *H. pylori*. Gerbils were challenged by a clinical isolate that possessed *vacA* and *cagE* and its isogenic mutants. The wild-type and *vacA* knockout mutants induced severe gastritis, whereas the *cagE* knockout mutant barely induced any gastritis. Similarly, no ulcers or gastric cancers were found in the gerbils infected with the *cagE* knockout mutant. The authors concluded that *cagE*, which forms a part of *cag* PAI, may play an essential role in the establishment of *H. pylori*-induced gastric lesions, including gastric carcinoma.

Gnotobiotic Piglet Model

Germ-free or gnotobiotic piglets are susceptible to colonization by *H. pylori*. Such animals delivered by Caesarian section into sterile isolation units have been challenged with *H. pylori* obtained from clinical isolates and maintained in germ-free conditions, during which time the extent of colonization and gastritis were monitored.^{36,37,71} These animals are susceptible to several different human-derived isolates, and colonization can be achieved at doses ranging from 10^3 to 10^6 CFU/10 mg gastric tissue. Bacterial strains adapted to challenge these piglets tend to increase their colonization efficiency, and piglets remain infected for over 3 months. Microscopically, polymorphonuclear cells may be present early in infection. Chronic active gastritis characterized by lymphocytes, plasma cells, and mucosal and submucosal lymphoid follicles in the lamina propria also occurred and increased in intensity with time. Gastric ulcers have been reported in infected piglets.⁷² However, this model has limitations for the study of *H. pylori* infection owing to the difficulties in maintaining the piglets within isolators for long-term studies. A proposed advantage of the gnotobiotic piglet model could be the correlation between development of inflammatory reactions and effects of intestinal microflora.

Other Animal Models

Gnotobiotic rats given *H. felis per os* on two successive days may develop gastritis.⁷³ Histological findings are quite similar to those in *H. pylori* gastritis in some adults and most children. Some SPF laboratory cat colonies do not harbor endogenous *H. felis*-like bacteria.⁷⁴ These cats are susceptible to *H. pylori* by experimental infection, which results in persistent colonization for 7 months, exhibiting chronic lymphofollicular gastritis in the gastric mucosa.⁷⁵ Ferrets and mink are naturally colonized with *H. mustelae*, and its natural infection results in chronic gastritis characterized by lymphocytic and plasmacytic inflammation in the gastric mucosa.⁷⁶ As described previously, ferrets have been used to evaluate the role of *Helicobacter* in gastric carcinogenesis associated with cocarcinogenic agents. Nine of 10 infected ferrets developed gastric carcinoma following a single oral dose of 50 to 100 mg of MNNG.⁶⁵

DIFFERENCES BETWEEN *H. PYLORI*-INFECTED ANIMAL MODELS AND HUMAN DISEASE

The colonization patterns of *Helicobacter* spp. in animal models differ from those seen in humans. Even in gastritis, which is the commonly reproducible lesion in animal models, the pathological features do not mimic the lesions seen in humans. Neutrophil activity is rarely seen in the animal models (especially rodent models), and there is no correlation with *cag* PAI state. The most severe gastritis in the mouse is seen with the *cagA* negative bacterium *H. felis*, and the inflammatory reactions are restricted to the gastric body mucosa away from areas of maximum

bacterial density. Other characteristics are severe gastritis and lymphoid follicular hyperplasia in the submucosal layer seen in the *H. pylori*-infected gerbil models. Such severe lesions are not observed in human gastritis. Gastric ulcers are rarely seen in the animal models and are not consistently reproduced by *H. pylori* infection alone, and duodenal ulcers have not been reported in any of the animal models of *H. pylori* infection. The only animal model that does develop gastric ulcers consistently is the *H. pylori*-infected Mongolian gerbil. Therefore, the pathogenesis of peptic ulcers does not associate only with *H. pylori* infection and may reflect in part other host (human) factors (for example, gastric physiology, anatomical characteristics, psychological features). Of course, gastric carcinogenesis in *H. pylori*-colonized gerbils raises several questions about special characteristics of this animal model (lack of immunological reagents, lack of transgenic/knockout strains). These phenomena are intrinsic in interpreting animal models for any disease and are their inherent limitations. Caution must be exercised when extrapolating from any animal studies to the human disease state.

FUTURE PERSPECTIVES

The possession of two complete genome sequences of *H. pylori* provides us a new approach to understanding the mechanisms of colonization, pathogenesis, and protection by studies of animal models with isogenic mutants.^{77,78} Although the SS1-infected mouse model is widely used for analysis of this bacterium, there are some difficulties in producing an isogenic SS1 mutant (such as *cag* PAI). In the near future, target gene expression *in vitro* will be analyzed via several genetic technologies, and such mutant strains adapted to animal models *in vivo* will reveal new evidence about this important gastric pathogen.

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CHAPTER 9

Animal Models for Tropical Parasitic Diseases

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FILARIASIS

Introduction

Filariasis is a parasitic disease caused by parasitic nematodes of the family Filariidae. Three species are of significance, *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*.¹ Transmission is via the bite of blood-feeding female mosquitoes, which transmit immature larval forms of the parasitic worms from human to human. Filariasis is endemic in over 80 countries in Africa, Asia, South and Central America, and the Pacific islands. More than 40% of all infected people live in India, and one third live in Africa.¹ *Wuchereria bancrofti* is distributed throughout the tropical regions of Asia, Africa, China, the Pacific, and the Americas.² *Brugia malayi* is confined in distribution to South and South-East Asia, India, and Korea, while *B. timori* is found in Timor and parts of Indonesia.

Etiology and Transmission

Wuchereria bancrofti parasites are mainly transmitted by *Culex quinquefasciatus* mosquitoes and some species of *Anopheles*. *Brugia* parasites are mainly transmitted by *Mansonia* mosquitoes. In humans, adult worms can live for many years, producing large numbers of larval forms (microfilariae), which circulate in the lymphatic system and blood, where they can be ingested by

blood-feeding mosquitoes, completing the transmission cycle.¹ Adult *W. bancrofti* are threadlike white worms. The male (4 cm × 0.1 mm) is coiled with a corkscrewlike tail and two spicules, the larger measuring 500 µm.² The female (6.5 cm × 0.2 to 2.8 mm) has a tapering anterior end with a rounded swelling. Adults lie coiled together in lymphatic vessels and glands. Eggs lie in the upper uterus enclosed in a chorionic membrane, which becomes a sheath when the microfilariae are born viviparously. Microfilariae are sheathed and measure 280 × 7 µm. One fifth of the length from the head is a V-shaped patch known as the “anterior V spot” and a short distance from the tail is the “posterior V spot.”²

The genus *Brugia* has eight species but only *B. malayi* and *B. timori* cause infection in humans, although *B. pahangi* has been transmitted to humans experimentally. Microfilariae of *B. malayi* are sheathed and measure 200 to 250 × 5 to 6 µm and have two isolated nuclei at the tip of the tail, while they lack nuclei in the cephalic space.² There are different strains of these filarial parasites depending on the periodicity of the microfilariae in the peripheral blood. Periodicity is a biological rhythm inherent in the microfilariae but influenced by the circadian rhythm of the host, which involves changes in the oxygen tension between venous and arterial blood by day and night and changes in the body temperatures. They are classified as:

1. Nocturnally periodic, which appear in peripheral blood at night
2. Diurnally subperiodic, which are present in peripheral blood during the day and at night but in greater numbers during the day
3. Nocturnally subperiodic, which are found both during the day and at night but in greater numbers during the night²

The adult worms, male and female, live in the lymphatics, where they can survive for 10 to 18 years. Microfilariae are produced from ova in the double uterus of the female, and unsheathed microfilariae appear in peripheral blood 6 months to 1 year after infection. They are then ingested by blood-feeding mosquitoes. Microfilariae, which can survive for 5 to 10 years in the absence of reinfection, undergo three stages of development in the insect host to form the infective larvae. These are injected by the female mosquito while feeding on another host.²

Pathogenesis and Clinical Manifestation

Infective larvae develop into adult worms in the afferent lymphatic vessels, causing severe distortion of the lymphatic system.¹ The earliest changes occur 4 weeks after infection.³ Pathological changes in the lymphatics are a result of immunological reactions by the host. Within 4 days of the infective larvae reaching the lymphatics, there is marked cell-mediated response in the regional lymph glands followed by an antibody-mediated response in the afferent lymphatics caused by an antimacrophilarial antibody. This response is the cause of local lymph gland enlargement in early filariasis. Disturbance of the lymph flow with lymphoedema results only when a strong resistance to reinfection has occurred with appearance of an antimicrofilarial antibody. The acute inflammatory changes induced by these processes are first an infiltration of polymorphonuclears, histiocytes, and many eosinophils, with a few lymphocytes in and around the lymphatics, followed later by death of the adult worm with epithelioid granuloma and foreign body giant cells. Dead worms calcify or become lysed and surrounded by fibrosis. Lymphatic abscesses may form at the site of dead and degenerating worms. The lymphatics are finally obliterated by fibrosis and microfilariae disappear from the blood.² There are three basic stages of the disease:

1. *Asymptomatic*: Patients have hidden damage to the lymphatic system and kidneys.
2. *Acute*: Attacks of filarial fever (pain and inflammation of the lymph nodes and ducts, often accompanied by fever, nausea, and vomiting) increase with severity of chronic disease.
3. *Chronic*: May cause elephantiasis and hydrocoele (swelling of the scrotum) in males and enlarged breasts in females.

Animal Models

Mice

Severe combined immunodeficient (SCID) mice, which cannot generate functional B or T lymphocytes, have been used for studies of filariasis.⁴ They are immunodeficient, and they have been reported to be receptive to reconstitution with human immune cells. Nelson and collaborators⁴ injected them with the infective larvae of *B. malayi* and after 6 weeks, the worms were observed in various stages of development in 90% of the mice. By 8 weeks and thereafter, microfilariae were detected in blood and in the peritoneal cavity in 52% of the mice. This suggests that the SCID mouse is an important model of filariasis. In another study,⁵ microfilaria of *B. malayi* were injected intravenously into mice. The level of microfilaremia was found to be proportional to the number of parasites injected, with approximately 1 to 3% of microfilaria being found in the peripheral circulation. Other findings have indicated that susceptibility of mice to *B. malayi* is strain associated.⁶ Laboratory mice have been infected with *Litomosoides sigmodonti*.⁷ They accommodate the full developmental cycle of the parasite. The disadvantage is that this parasite is not pathogenic to man. To determine the role of B-lymphocytes in antifilarial immunity, the murine peritoneal cavity has been used as a model, using *B. pahangi*.⁸ It was found that B-lymphocytes are required for clearance of primary and challenge infections of *B. pahangi* third-stage larvae.

Leaf Monkeys

Clinical trials of ivermectin and diethylcarbamazine treatment in leaf monkeys have been carried out.⁹ The monkeys were infected with *Wuchereria kalimantani*. The optimal effect occurred at 200 mg/body weight. To evaluate the effect of the preadult stage parasite and multiple timed exposures to infective larvae in the development of limb edema caused by *B. malayi*, leaf monkeys have been used.¹⁰ Infection was initiated with subcutaneous inoculation of infective third-stage larvae (L3). The preadult stage was found to be the most potent inducer of limb edema.

Indian leaf monkeys have also been used in assessment of cellular immune responses in asymptomatic and symptomatic animals infected with *B. malayi*, where the effect of CD4 and CD8 T cells in disease progression was assessed.

Rhesus Monkeys

To investigate whether *Wolbachia*, which is a symbiotic bacterium living within filarial nematodes, may be involved in disease progression, *Wolbachia*-specific immune responses were assayed in a group of *B. malayi*-infected rhesus monkeys. Serum IgG antibodies specific for a major *Wolbachia* surface protein were detected in two of the 12 animals.¹¹ A positive correlation of T helper 1 cytokines and lack of induction of IL-2R+ T cells with T-cell unresponsiveness in rhesus monkeys infected with *B. malayi* has been demonstrated.¹²

Mandrills

Parasitological and immunological effects induced by immunization of mandrills against filarial *Loa loa* have been studied using infective stage larvae irradiated at 40 krad.¹³ Six mandrills were immunized with 150 parasites (infective larvae) and then challenged with 100 L3, 60 days after the initial immunization. The outcome was compared to that in six mandrills infected with the normal L3. No clear association was found between vaccination and microfilaremia until day 245, when a significant drop in the level of microfilariae occurred in the vaccinated animals as compared to the rest of the animals. Cellular responses to *Loa loa* experimental infection in mandrills vaccinated with irradiated infective larvae have been investigated.¹⁴ All animals were challenged

with 100 intact L3 (day 0). It was noted that vaccination delayed the appearance and mean level of microfilaremia.

Other Animal Models

Birds were infected subcutaneously with infective L3 larvae of *B. malayi* and evaluated as an animal model for assessing microfilaricides by means of observing the change in microfilariae density but not by worm recovery.¹⁵ Filariasis due to *Pelecitus* was found in the subcutaneous tissue of the neck of a domestic pigeon from Spain that died from trichomoniasis.¹⁶ Filariae were observed in the congested and hemorrhagic cervical connective tissue. No inflammation was observed, however. Rabbits have been used to establish animal models for dirofilariasis.¹⁷ Rabbits were infected with the immature fifth-stage worms of *Dilofilaria immitis*. In the lungs, histopathological changes were observed, which were encapsulated by a fibrous wall.

Studies were carried out at monthly intervals on five dogs during the first 18 weeks following infection with 400 *Brugia pahangi* infective larvae in the left rear paw. Clinical signs, body temperatures, and microfilariae counts were also monitored during this time.¹⁸ The severity of the lymphatic pathology correlated with the clinical signs of limb edema and low microfilariae counts. *Wuchereria bancrofti* in Mongolian gerbils and hamsters infected with third-stage larvae showed that the worms were usually recovered from the testis, pelt, heart, and lungs.¹⁹ The parasite developed similarly in both models.

The prevalence of *D. immitis* in cats in Japan has been studied since 1957.²⁰ The surveys evaluated both stray and house cats, and the worms were detected at necropsy. *Millardia meltada* were infected with *Acanthocheilonema viteae* and examined for susceptibility. The morbidity of the infected animals was low as compared to that of birds.²¹ However, these animals developed microfilaremia.

Immunology

Human adults not previously exposed to infection react more vigorously to infection than those who have been exposed since childhood, and the clinical picture of early filariasis is different.² Humoral immune mechanisms directed against adult worms are responsible for the damage to the lymphatics and eventually clear the microfilariae from the blood, which accounts for the fall in microfilaremia with age. In an experiment to demonstrate the role of the humoral immune responses of Indian leaf monkeys to *B. malayi*,¹⁰ it was noted that while both the circulating immune complexes and filarial-specific IgG levels were comparable in animals showing no disease symptoms (asymptomatics) and some animals showing symptoms (symptomatics), IgG levels peaked during the prepatent stage in symptomatics and in the latent stage in asymptomatic animals in contrast to the symptomatic animals. An immunoblot analysis showed nonreactivity of 17 and 55 kDa antigens with sera of symptomatic animals. The results thus suggest that humoral immune responses as measured in this study do not precede the development of manifestations. The relationship between parasitological status and humoral responses to *L. loa* antigens in the mandrill model after immunization with irradiated L3 and infection with L3 was assessed.²² The plasma of these animals was analyzed by Western blot using adult, microfilaria, and L3 antigens. The plasma of all animals recognized several antigens with molecular weights varying from 13 to 120 kDa.

The relationship between antigen-specific responsiveness, parasitic burden, and lymphatic pathology was investigated in nine rhesus monkeys with chronic *B. malayi* infections.¹² The data showed that diminished production of Th1 cytokines and lack of induction of IL-2R+ T cells might contribute to the unresponsiveness in monkeys nonresponsive to filarial antigen. It also showed that polarization of immune responses and lymphatic pathology observed in rhesus monkey is similar to that genetically described in human filariasis patients. In another experiment to determine the immune response elicited by various stages of the parasite,¹⁰ the preadult stage of

the parasite was found to be the most patent inducer of limb edema, followed by L5 and L4. The presence of proinflammatory cytokines tumor necrosis factor alpha, interleukin-1 beta and interleukin-6 in the edema fluid in the leg receiving the parasite challenge indicated that the limb edema development was due to parasite-mediated cytokine responses.

Cellular immune mechanisms are responsible for the death of microfilariae, which occurs in some people and is responsible for tropical eosinophilia.² In symptomatic Indian leaf monkeys, leucocyte migration inhibition responses to homologous adult parasite antigen were significantly suppressed as compared to asymptomatic monkeys.²³ When compared with asymptomatic monkeys, CD8 T cells in monkeys showed a significant increase after day 180 post inoculation. CD4 T cells remained in the normal range until day 200 post infection, after which they showed a marginal increase. B cells have been demonstrated to be important in the production of Th2 type cytokines by peritoneal cells of mice.⁸ B-cell-deficient mice have a defect in inflammatory cell recruitment to the peritoneal cavity following *B. pahangi* infection. Specific T-cell hyporesponsiveness and depressed antibody production are a key feature in human infection with filarial nematodes.²⁴ There are unusually high levels of specific IgG4. During chronic filariasis, parasite-specific cellular responsiveness is profoundly downregulated.²⁵ Cystatins, a group of cysteine protease inhibitors, have been implicated in this suppressive activity. It has also been demonstrated that recombinant interleukin-12 suppresses pulmonary eosinophilia and airway hyperresponsiveness by modulating T helper response in the lungs, with elevated interferon gamma and decreased IL-4 and IL-5 production.²⁶ Infections of humans with filarial parasites have long been associated with the maintenance of a dominant Th2 type host immune response. This is reflected in increases in interleukin-4 and -5 producing T cells, elevated IgE and IgG4 levels, and a pronounced eosinophilia.²⁷

LEISHMANIASIS

Leishmaniasis is the term used for a group of infectious diseases caused by trypanosomatid protozoans of the genus *Leishmania*, which comprises different species, all of which are obligate intracellular parasites of the mononuclear phagocytic cells of vertebrates. About 20 species and subspecies of the genus *Leishmania* are known to infect humans and cause a spectrum of diseases. Leishmaniasis causes considerable morbidity and mortality, particularly in developing countries. The disease may occur as an epidemic but more often it is endemic. The endemicity of the disease has been confirmed or suspected in 97 countries in southern Europe, Africa, the Middle East, the Indian subcontinent, and Central and South America.

Leishmaniasis affects an estimated 12 million people with more than 400,000 new cases per year.^{28–30} It is estimated that approximately 350 million people are at risk for leishmaniasis worldwide with a yearly incidence of 300,000 cases of cutaneous leishmaniasis (CL) and 100,000 cases of visceral leishmaniasis (VL).^{31–33} However, studies have indicated that these estimates are significantly lower than the actual numbers, primarily due to lack of reporting and inadequate health surveillance.³³

Etiology and Transmission

Leishmania parasites are dimorphic with two developmental stages: promastigotes (Figure 9.1A) and amastigotes (Figure 9.1B).

Promastigotes are slender and elongated forms of the parasite measuring about 10 to 20 μm , with an anteriorly located flagellum. This stage of the parasite is found in the gut of the arthropod vector and is the form that is inoculated into the skin of the vertebrate host.³⁴ It is also the form that is easily grown in cell-free cultures. The amastigotes are oval-shaped forms of the parasite measuring 3 to 7 μm that are found in the macrophages of the vertebrate host. The flagellum is

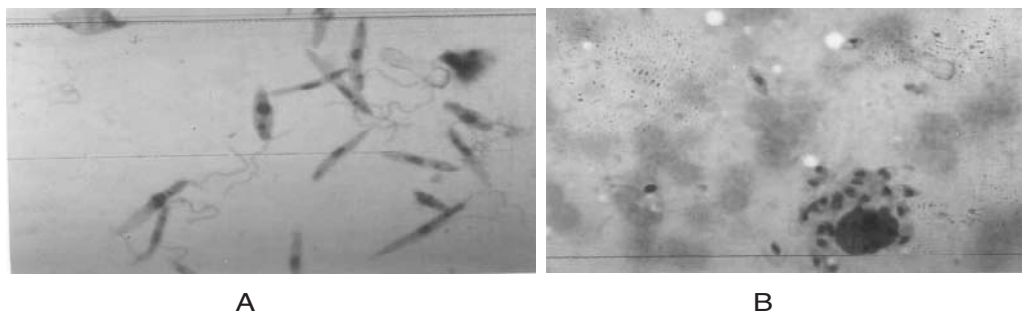


Figure 9.1 A. *Leishmania major* promastigotes propagated in Schneiders' drosophila medium. B. *L. major* amastigotes, extracellular and intracellular amastigotes demonstrated. Preparation from liver biopsy of an infected BALB/c mouse. Both preparations were Giemsa-stained. Original magnification, $\times 100$.

reduced to a small dot, referred to as a kinetoplast.³⁴ This stage of the parasite is also known as Leishman–Donovan (LD) bodies, which are named after their discoverers.^{35,36} Both forms of the parasite multiply by binary fission. In macrophages of mammalian hosts, amastigotes multiply and eventually rupture the cell and invade more macrophages. The disease is spread through a vector, a phlebotomine sandfly of the genera *Phlebotomus* (Old World) or *Lutzomyia* (New World). The sandfly breeds in warm humid microclimates and is typically found in rodent burrows, termite hills, and organic wastes such as cow dung and rotting vegetation.³⁷ Disease transmission occurs when female sandflies ingest amastigotes while taking a blood meal from an infected person or a reservoir host. These transform into promastigotes within the insect's gut, migrate to the proboscis, and are inoculated into the dermis of the new host during a blood meal. Direct person-to-person transmission of leishmaniasis has occasionally been described (blood transfusion, sexual and congenital transmission, and syringe exchange among intravenous drug users).³⁸ The promastigotes invade the macrophages, transform into amastigotes, and multiply within the macrophages of the skin, mucous membrane, and viscera.³⁹ Most forms of leishmaniasis are zoonotic, and humans are infected only secondarily. Animal reservoirs of species pathogenic to man include dogs, foxes, rodents, gerbils, hyraxes, and sloths.⁴⁰

Clinical Manifestations

Infection with *Leishmania* in humans results in a spectrum of diseases depending upon the species involved and the efficiency of the host's response to the parasites.^{41,42} Cutaneous leishmaniasis, caused by *L. major* and *L. tropica*, is characterized by skin ulcers that heal spontaneously, leaving scars. Diffuse cutaneous leishmaniasis caused by *L. aethiopica* and *L. mexicana* causes widespread thickening of the skin with lesions resembling lepromatous leprosy, which do not heal spontaneously. Infection with *L. braziliensis* results in mucocutaneous leishmaniasis due to spread of organisms to mucosal sites from a primary cutaneous lesion established earlier. Metastatic spread may occur to the oral, nasal, and pharyngeal mucosa, causing disfiguring leprosy-like tissue destruction and swelling. Recent studies in the Sudan indicated that mucocutaneous leishmaniasis might be caused by *L. donovani*.⁴³ Visceral leishmaniasis or kala-azar is caused by *L. donovani*, *L. infantum*, and *L. chagasi*. The infection spreads internally to involve cells of the reticuloendothelial system, primarily the spleen, liver, and bone marrow. Common symptoms include fever, malaise, weight loss, coughing, and diarrhea accompanied by anemia, skin thickening, and hepatosplenomegaly.^{43,44} Visceral leishmaniasis is fatal if untreated due to failure of the host to mount an effective protective immune response, profound cachexia, secondary infection, and hemorrhage.⁴⁴ Visceral leishmaniasis may occur as sporadic cases or in periodical epidemic waves in the areas where it occurs.^{45,46}

Animal Models

Much of the current knowledge in leishmaniasis has been generated through experimental animal studies. Experimental animal models for leishmaniasis are useful to provide the means for *in vivo* maintenance of virulent strains of parasites, production of amastigotes to study the pathogenesis of leishmaniasis and antileishmanial immunity, and to test antileishmanial drugs and vaccines.⁴⁷ In experimental conditions, *Leishmania* parasites are capable of inducing an infection in a wide variety of mammals, many of which would not be natural hosts for the parasites. Nevertheless, animal models are expected to mimic the pathological features and immunological responses observed in humans when exposed to a variety of *Leishmania* species with different pathogenic characteristics. It is noteworthy that many experimental models have been developed, each with specific features, but none accurately reproduces what happens in humans or dogs. A major difference between natural and experimental infection is the parasite inocula. In natural conditions, the infected sandfly vector deposits a few hundred metacyclic promastigotes in the dermis of the host, whereas experimental infections are usually induced by the injection of millions of promastigotes grown in axenic cultures *in vitro* or amastigotes recovered from cutaneous lesions or infected spleens.^{34,47–49} However, the relevance of experimental models in understanding the immunopathogenesis of leishmaniasis, designing and formulating *Leishmania* vaccines, and testing antileishmania drugs cannot be underrated. Some of the experimental animal models widely used for study of leishmaniasis include the Syrian golden hamster, mouse, cotton rat, opossum, armadillo, ferret, dog, and Old and New World nonhuman primates.^{47,50–53}

Murine Models and Immunology of Leishmaniasis

Studies in animal models, especially mice, have created an improved understanding of pathogenesis and protection during infection with *Leishmania* parasites.^{54,55} Mice of different phenotypes, of strains that are either susceptible or resistant to *Leishmania major*, have contributed immensely to the current understanding of leishmaniasis. A good example of a susceptible strain is BALB/c and of a resistant strain (negative model) is C57BL/6. In addition to the susceptible and resistant strains of mice, there are transgenic mouse models, which have been used to add knowledge to the current understanding of the immunology of leishmaniasis. Examples of transgenic mouse models are interferon gamma (IFN-gamma), IL-4, CD8⁺, and CD4⁺ knockout mouse models.⁵⁵ Immunopathogenic and immunoprotective mechanisms operating in leishmaniasis have been extensively studied in murine models. Good evidence has demonstrated the pivotal role of CD4⁺ T cells in the control of the infection including the finding that Th1 cells are associated with resistance, whereas Th2 cells are required for disease progression.^{54,55} The principal immunological effector mechanisms responsible for the control of *Leishmania* parasites are the activation of macrophages by IFN-gamma and the generation of reactive oxygen products and nitric oxide.^{56–58} In addition to IFN-gamma, macrophage activation requires signals provided by tumor necrosis factor- α (TNF- α).^{59,60} As might be expected from the intracellular location of *Leishmania* organisms, the predominant T cell that is activated during leishmaniasis is a CD4⁺ T cell. Using CD8⁺ knockout mice, it has been shown that resistance can occur in the absence of CD8⁺ T cells, but not in the absence of Class II restricted T cells.⁵⁴ Interestingly, CD4⁺ appears to be able to mediate both resistance and susceptibility.^{61,62} Studies conducted in mice during infection have demonstrated that IL-4 is important for Th2 cell development, while IL-12 and IFN-gamma promote Th1 cell development.⁶² IL-12 initiates cell-mediated immunity and Th1 cell development during development of an immune response to many pathogens. In *L. major*-infected C3H mice, IL-12 is evident during the first 48 h of infection, and the production depends on upregulation of CD40L on T cells and stimulation of CD40-expressing cells such as macrophages and dendritic cells. Consistent with the importance of IL-12 in Th1 development is the finding that IL-12 can facilitate Th1 cell development in a *i* vaccine model. The efficacy of exogenous IL-12 contrasts with IFN-gamma,

which is unable to permanently induce a Th1 response. Thus, Th1 cell development in C3H mice occurs via a pathway involving IL-12, which subsequently activates natural killer (NK) cells to produce IFN- γ , and both IL-12 and IFN- γ are required for development of a Th1 response.

Hamster and Dog Models

Dogs are natural hosts of *L. infantum* and *L. donovani* and good models for pathogenesis for visceral leishmaniasis. They have been widely used for testing antileishmania agents. Syrian golden hamsters are highly susceptible to *Leishmania* species responsible for visceral leishmaniasis. This animal model has been widely used to study the disease pathology and for testing antileishmania agents. However research in hamster models for leishmaniasis has been hampered by lack of immunological reagents.^{63–66}

Nonhuman Primate Models

Some of the observations made in murine models might not be similar or relevant to human hosts due to the distance in phylogeny. The development of a nonhuman primate model of leishmaniasis, which largely mimics the human situation, is desired for studies of different aspects of the disease that would not be possible in humans for ethical reasons. This would also complement studies in other model systems. However, for financial and ethical reasons, the use of primates in biomedical research is limited. Studies involving these animals have therefore been tailored to solve questions that cannot be answered in other animals. Monkeys are normally the final experimental animals to be used in studies of the safety and efficacy of vaccines and drugs developed in other laboratory animals.

Many primates have been experimentally infected with different *Leishmania* species, including the marmoset, the vervet monkey, the baboon, the squirrel monkey, and the Indian langur. The langur is a suitable model for visceral leishmaniasis,⁶⁷ while the squirrel monkey was reported as a model for South American visceral leishmaniasis.⁶⁸ The owl monkey (*Aotus trivirgatus*) was identified as susceptible to experimental infection with *L. braziliensis panamensis*,⁶⁹ the cause of South American cutaneous leishmaniasis. In other studies, the marmoset (*Calithrix penicillata*) and the squirrel monkey were shown to be susceptible to *L. braziliensis* and *L. panamensis*, respectively.^{70,71} Similarly, rhesus monkeys have been demonstrated as good experimental models for *L. amazonensis*.^{72,73}

A natural infection of the vervet monkey with *L. major*, the causative agent of human cutaneous leishmaniasis, was first reported in 1987.⁷⁴ Based on this finding, tests were conducted to establish whether vervet monkeys could be developed as an experimental model for cutaneous leishmaniasis. Intradermal inoculation of 10^6 to 10^7 *in vitro*-derived *L. major* promastigotes produced nodules of variable sizes in all animals within about 1 week, which progressed to ulcers and finally self-cured in about 120 days (Figure 9.2).⁷⁴ The results were similar to reports in patients suffering from natural cutaneous leishmaniasis with respect to lesion sizes and the time taken to cure.⁷⁵

Previous work had demonstrated that injection of sandfly salivary gland lysate from *Lutzomyia longipalpis*, a vector for visceral leishmaniasis in South America, could enhance infectivity in mice and produced cutaneous ulcers five to ten times larger than controls.⁷⁶ A similar experiment in vervet monkeys demonstrated that injection of the vector sandfly (*Phlebotomus duboscqi*) salivary gland lysate plus *L. major* promastigotes resulted in ulcers identical to those produced by infection without the lysate.⁷⁷ However, the number of parasites required to initiate the infection was less by about half, compared to controls. The time taken to self-cure was similar in experimental and control animals and to earlier experiments where no lysate was used.^{77,78} These results confirmed the reports in the murine model and extended it to the monkey model of cutaneous leishmaniasis using salivary gland lysate from the natural vector (*P. duboscqi*).



Figure 9.2 Cutaneous lesion (ulcerated nodule) on right eye brow ridge of a vervet monkey infected with 1×10^6 virulent *L. major* promastigotes from salivary gland lysate (SGL) from five pairs of 3-day-old female *Phebotomus dubosqui*. Photograph taken on day 56 post infection. Caliper in place measuring the nodule.

Following an infective bite by a female sandfly, the development of cutaneous leishmaniasis in human hosts normally starts by nodule formation, which progresses to ulcers and finally self-heals. The time taken to self-healing can be variable and may take years depending on host and parasite factors.^{41,42}

Experimental transmission of *L. major* parasites to rodents and humans by bites of *P. dubosqui* sandflies had previously been performed successfully.⁷⁹ Similar studies conducted in vervet monkeys and baboons demonstrated that satellite nodules indicative of where the flies had probed formed 2 weeks after an infected sandfly bite. The nodules developed central crusts, then ulcers, which coalesced with progressive disease to form single ulcers. The time taken from infection to self-cure and variability of the lesion sizes were similar to that in animals infected by needle injection. As with needle infection, all animals infected by sandfly bites developed cutaneous ulcers.^{48,78}

Establishment of a vervet monkey model for cutaneous and visceral leishmaniasis would be advantageous for the study of the two diseases. Studies conducted with other *Leishmania* have demonstrated that the vervet monkey is susceptible to *L. donovani* and *L. aethiopica*.^{78–83} Use of primate models for testing potential *Leishmania* vaccines is not widespread, as might be expected because primates are expensive laboratory animals that are difficult to obtain and to handle. Furthermore, T-cell markers and cytokine assays for nonhuman primates are limited, and this problem is compounded by considerable variation among individual animals, arising from their outbred nature.^{84,85} Currently, vervet monkeys and rhesus monkeys are being used to test potential *Leishmania* vaccines and diagnostic molecules.^{73,86–89}

Table 9.1 summarizes the animal models for leishmaniasis.

MALARIA

Malaria is a parasitic disease caused by the intracellular protozoan organism *Plasmodium* (phylum Apicomplexa, genus *Plasmodium*). There are 120 known *Plasmodium* species infecting humans, rodents, bats, avians, reptiles, and nonhuman primates.⁹⁰ Four species, *P. falciparum* (malignant tertian), *P. vivax* (benign tertian), *P. malariae* (quartan malaria), and *P. ovale* (ovale

Table 9.1 Summary Table of Animal Models for Leishmaniasis

Animal Species	Examples	Main Strength
Mice	BALB/c	Immunology, vaccines, chemotherapy
	C57BL/6	Negative model — immunology, vaccines, chemotherapy
	Transgenic mice	Immunology
Hamsters	Syrian golden hamster	Pathology, chemotherapy
Dogs	Different breeds	Pathology, vaccines, chemotherapy
Nonhuman primates	Langurs, vervet monkeys, rhesus monkeys, mandrills, owl monkeys, baboons, marmosets, squirrel monkeys	Vaccines, pathogenesis, chemotherapy, pathology

malaria), are known to parasitize humans and are transmitted by female mosquitoes of the genus *Anopheles*.⁹⁰ *Plasmodium falciparum* and *P. vivax* are the two most prevalent species causing human malaria. *Plasmodium falciparum* is the most lethal, accounting for over 90% of malaria-associated deaths. *Plasmodium vivax* accounts for approximately 4%, while *P. malariae* and *P. ovale* each account for less than 1%.^{90,91}

Malaria has been known as a human disease since the beginning of recorded history, but it was not until 1880 that Laveran discovered the parasite in the blood of malaria patients in Algeria.⁹² The disease continues to be a major cause of morbidity and mortality in approximately 40% of the world's population, mainly inhabitants of developing countries in the tropical and subtropical regions of the world. Annually, it is estimated that 300 to 500 million people suffer from the disease and up to 2.7 million die. Africa accounts for over 90% of the deaths, with childhood mortality approximated at 2 million per year.⁹³ Because of its high morbidity and mortality rates, especially in children under 5 years and pregnant women due to severe anemia, malaria exerts a heavy toll on people living in endemic areas.⁹⁴

Animal Models

The value of animal models in malaria research has long been appreciated.⁹⁵ Different species of animals have been used to study malaria parasite biology, immunology, molecular biology, immunopathogenesis, chemotherapy, and vaccine development for prevention and control of disease in the human. *Plasmodium falciparum* and *P. vivax*, the two most prevalent species of human malaria, have restricted host ranges,⁹⁶ which limits research on parasite biology, especially at the host–parasite interface. This has necessitated the development of experimental systems to model complex interactions between parasite and host.¹⁰¹ Rodents and nonhuman primates are the main experimental models that have been used in understanding malaria parasite biology.^{97–99}

Rodent models are well established in studies of malaria^{100,101} and have contributed immensely to our understanding of various aspects of the immune responses. However, their relevance to malaria in humans is questionable because of the many differences between rodent species and humans.¹⁰² An effective animal model for human malaria disease should closely mimic the disease in humans. Nonhuman primates are ideal candidates because they are susceptible to many species of *Plasmodium*¹⁰³ and have humoral and cellular responses similar to those of humans.¹⁰² Among the nonhuman primates being used for malaria research, macaques, *Aotus* monkeys, and chimpanzees are well-established models.^{98,104} Although baboons are used in the study of important human parasitic diseases,¹⁰⁵ the baboon malaria model is underdeveloped compared to models based on other nonhuman primates.

Rodent Models

Rodent models of malaria have been utilized to study clinical disease as well as parasitology, immunology, chemotherapy, and vaccination.

Parasitology and Clinical Aspects

Mouse models have been used for studies of acute and chronic presentations of malaria infection, such as cerebral malaria which is a serious and often fatal complication of *P. falciparum* infections.^{97,106} The precise mechanisms involved in the onset of neuropathology associated with cerebral malaria remain unknown, but parasite sequestration in the brain, metabolic disturbances, and host immune responses are all thought to be involved. Studies have revealed changes in the infected host that are consistent with a systemic inflammatory response associated with cerebral malaria.¹⁰⁷ It has been proposed that endothelial cell adhesion molecules (ECAMs) contribute to the adhesive interactions of *Plasmodium*-infected erythrocytes and immune cells with vascular endothelial cells. Radiolabeling techniques have quantified the expression of different ECAMs (ICAM-1, VCAM-1, P-selectin, E-selectin) in different regional vascular beds of *Plasmodium berghei* ANKA-infected mice (PbA), a well-recognized model of human cerebral malaria. The roles of T lymphocytes and certain cytokines (TNF-alpha, IL-12, IFN-gamma) in mediating the infection-induced expression of ICAM-1 and P-selectin were assessed by using relevant mutant mice.¹⁰⁷ These studies indicated that vascular endothelial cells in most regional circulations assume an inflammatory phenotype and that cytokines and immune cells mediate this response in a tissue-specific manner. A recent review by de Souza and Riley⁹⁷ has outlined the current state of knowledge of cerebral disease in humans, and discusses the contribution of studies of animal models to elucidation of the underlying mechanisms.

It was recognized early on that the study of human malaria was hampered by the lack of small animal models for the human-infecting malarial parasites. Thus studies focused on *in vitro* adaptation of the erythrocytic stages of the human malarial parasite *P. falciparum*. This was achieved in the presence of ascites fluid from mice homozygous for the SCID mutation and was the first demonstration of human malarial parasite propagation in mice and transmission of these parasites to the invertebrate vector.¹⁰⁸

Severe anemia is a major cause of death in falciparum malaria. Blood transfusion increases survival in humans and in animal models of this disease. Rats have been used to study the effects of transfusions of modified hemoglobin in rats with high-grade parasitemia. Modified hemoglobin decreased lactic acidosis and corrected anemia as well as transfusions with red blood cells did; these findings may correlate with improved survival and suggest a possible proerythropoietic effect.

The use of murine models is increasing our comprehension of the genetics of host resistance to malaria, which is essential to understanding the complex host/parasite interaction as reviewed by Burt.¹⁰⁹ Current research is directed towards the genetic dissection of both the murine and human host responses to the disease. Significant progress has been made toward the mapping of novel murine resistance loci. In addition, the role of the major histocompatibility complex in the host response has been examined in both animal models and human populations. The cloning of genes involved in malarial resistance using rodent models¹⁰⁹ will lead to a greater understanding of this complex disease in humans¹¹⁰ and potentially to the development of more effective medical intervention.

Immunology

Immune responses during malaria infection have been widely studied using mouse models of disease. The studies have focused on both arms of the adaptive immune response, i.e., antibodies and cell-mediated responses,^{111,112} as well as innate mechanisms.¹¹³ Antibody responses are important

in the development of acquired immunity to human malaria infections. Characterizing the relative specificities of antibody responses during the acquisition of immunity and in hyperimmune individuals is thus an important adjunct to vaccine research. This is logistically difficult to do in the field but is relatively easily carried out in animal models. Infections in inbred mice with the rodent malaria parasite *Plasmodium chabaudi chabaudi* AS represent a good model for *P. falciparum* in humans. This model has been used in a comparative analysis of cross-reactive and specific immune responses in rodent malaria using CBA/ca mice.¹¹⁴ Importantly, the antibody response included opsonizing antibodies, which bound to infected erythrocytes, leading to their phagocytosis and destruction by macrophages. These results were an important pointer to the role that antibodies to both variable and invariant antigens may play in protective immunity in the face of continuous susceptibility to reinfection.

The immune responses associated with blood stages of malaria infections have been extensively studied in mouse models¹¹⁵ and have elucidated the nature of cytokines involved in protection and pathology. Mouse models have also been used to investigate the immune responses associated with liver stages of *Plasmodium* infections. The liver stage of malaria is clinically silent but immunologically significant. Ample evidence exists for an effective CD8(+) T-cell response to this stage as well as the involvement of gamma-delta T cells and NK1.1(int) cells in immunized animal models.^{116,117} It has been reported that several host gene expressions in the liver, spleen, and kidney of BALB/c mice are altered during the liver stage of *Plasmodium yoelii* infection.¹¹⁶ These changes suggested that the immune responses to *P. yoelii* infection are both parasite and organ specific. Because *P. falciparum* is an intracellular pathogen, it is potentially susceptible to cytotoxic T-lymphocytes, a crucial component of the protective immune response to viral infections. Evidence from animal models points to a protective role for cytotoxic T-lymphocytes against *P. falciparum*,¹¹⁸ although cytotoxic T-lymphocytes specific for this pathogen have been difficult to identify in man.

The association of automimmune responses with malaria infection has long been recognized, and recent studies in mice have shed light on this clinical aspect of the disease. In Balb/c mice, CD4(+) T cells co-expressing CD25 [CD4(+)CD25(+) T cells] have been identified as immunoregulatory suppressors modulating autoimmune response.¹¹⁹ It was shown that CD25 depletion clearly delayed the growth of parasitemia during parasite challenge, particularly in immunized mice. These findings demonstrated that CD4(+)CD25(+) T cells are able to influence protective immunity underlying premunity to *P. berghei* NK65 parasites.¹¹⁹

Chemotherapy

New drugs against malaria are first screened in animal models, and even after they are licensed for human use, further studies can continue in laboratory animals. For example, the observation of the toxicity of artemisinin compounds in animals, but not in humans, was shown to be likely due to different pharmacokinetic profiles after different routes of administrations in mice and humans.¹²⁰ The use of rodents in *in vivo* assessment of drugs for malaria chemotherapy has been developed using Swiss mice infected with *Plasmodium vinckei petteri* with novel modes of drug administration.¹²¹ Another example is the study of effects of oral artesunate in *P. berghei*/mice and *P. knowlesi*/*Macaca mulatta* models, which provide a useful index for clinical trials.^{122,123}

The effects of intravenous chloroquine infusion or the topical application of a pectin hydrogel chloroquine matrix patch has been examined in rats. The results suggested that the pectin chloroquine patch matrix preparation has potential applications for transdermal delivery of chloroquine and perhaps in the management of malaria.¹²⁴ Other studies of chloroquine efficacy have been done in mice, revealing their ability to downmodulate proinflammatory cytokine responses.¹²⁵ Other studies have assessed the effectiveness of chloroquine analogs against chloroquine resistant *P. falciparum* in mouse models.¹²⁶ The *in vivo* assessment of iron chelators that inhibit *in vitro* proliferation of *P. falciparum* was performed on Swiss mice infected with *P. vinckei petteri* with novel modes of drug administration and release.¹²¹ Delivery of these drugs by polymeric devices

was particularly useful for treating *P. berghei* K173-infected C57Bl mice, a suggested model of cerebral malaria, in which classical methods of delivery were ineffective. A considerable number of iron (III) chelators, designed for purposes other than treating malaria, have antimalarial activity *in vitro*; apparently through the mechanism of withholding iron from vital metabolic pathways of the intraerythrocytic parasite, as has been reviewed.^{127–129} Several of the iron (III)-chelating compounds also have antimalarial activity in animal models of plasmodial infection, such as rodents. Through animal studies it was shown that iron chelation therapy with desferrioxamine, the only compound of this nature that is widely available for use in humans, has clinical activity in both uncomplicated and severe malaria in humans.¹²⁷

Vaccination

Vaccines have long been recognized as having the best possible chance of combating malaria in endemic countries.^{130–134} Characterizing the specific immune responses of individuals to malaria infection is an important adjunct to vaccine research. This is logistically difficult to do in the field but is relatively easily carried out in animal models. Immunization experiments in animal models by several investigators have suggested different strategies for vaccination against malaria. Studies of preerythrocytic vaccines for *P. falciparum* malaria, many of them conducted in rodent models, have recently been reviewed.¹³⁵ The vaccines targeting the preerythrocytic stages include peptide-based subunit molecules that have been tested in rodents.¹³⁶ Many of the targets from liver-stage malaria antigens have been shown to be immunogenic and to protect mice from the sporozoite challenge. CD8+ T cells have been implicated as critical effector cells in protection against the preerythrocytic stage of malaria in mice and humans following irradiated sporozoite immunization. Based on these experimental results, the development of a CD8+ T cell inducing vaccine has moved forward from epitope identification to planning stages of safety and immunogenicity trials of candidate vaccines, as recently reviewed by Oliveira-Ferreira and Daniel-Ribeiro.¹³⁷

Preclinical evaluation of synthetic peptides corresponding to the C-terminal regions of the circumsporozoite (CS) protein in various plasmodia showed that these preparations were immunogenic and safe upon injection in various animal models.¹³⁸ Moreover, the CS C-terminal peptide derived from *P. berghei* conferred protection upon challenge with live sporozoites in mice. A GLP preparation of the synthetic peptide was evaluated in an open, nonrandomized, Phase I human trial. Data obtained showed that the malaria vaccine was safe, was well tolerated, and gave rise to high antibody titre, CD4+, and CD8+ lymphocyte responses.¹³⁸ Rodent models have also been utilized to test the efficacy of subunit malaria vaccines formulated with novel adjuvants.^{139–141}

Immunization with DNA vaccines encoding relevant antigens can induce not only cell-mediated immune responses but also humoral immune responses against pathogenic microorganisms in several animal models. The safety, tolerability, routes of immunization, and associated immune responses of malaria DNA vaccines have been tested in rodent models.^{142,143} Tests of leading vaccine candidates against erythrocytic stages of malaria have been undertaken in rodent models.¹⁴³ It was demonstrated that when the C terminus (PyC2) of *Plasmodium yoelii* merozoite surface protein-1 (MSP-1) was expressed as a fusion protein (GST-PyC2) with glutathione S-transferase (GST), or given as a DNA vaccine, it elicited antibody-mediated protective immune responses in BALB/c mice.

Transgenic mice for studying immune responses to epitope vaccines have shown promise as future model for human malaria.^{144,145} Two strains of transgenic mice have been generated that secrete into their milk a malaria vaccine candidate, the 42-kDa C-terminal portion of *P. falciparum* merozoite surface protein 1 (MSP1(42)).¹⁴⁶ These studies demonstrated the potential for producing efficacious malarial vaccines in transgenic animals. Evaluation of the falciparum and vivax malaria panmalarial antigen (PMA) and merozoite surface protein 1 (MSP1) using mice infected with *P. berghei*, a rodent malaria, has increased the potential of rodents as models for malaria vaccine studies.^{147,148}

Nonhuman Primate Models

Nonhuman primates (monkeys and apes) represent a valuable resource for testing potential malaria vaccine candidates and drugs for human use. Safety and protective efficacy studies in animal models are critical steps for vaccine and drug development, and primate models are probably the most appropriate for this purpose.¹³⁸ In addition, studies in nonhuman primates have contributed to our knowledge of the biology of malaria host–parasite relationships.

Susceptibility

Plasmodium falciparum infection in humans leads to a variety of symptoms ranging from an influenza-like syndrome to life-threatening complications. Animal models are useful tools for detailed studies of host–parasite interaction and host factors contributing to the various clinical manifestations. In examining the different clinical, parasitological, and hematological parameters associated with *P. falciparum* infection, squirrel monkeys (*Saimiri sciureus*) have been shown to be a good model for exploring aspects of the host–parasite relationship in malaria.¹⁴⁹

Among the nonhuman primate models for human malaria, *Aotus* monkeys from South America are the most preferred.⁹⁸ Although the *Aotus* genus provides several species susceptible to both *P. falciparum* and *P. vivax*, and with different susceptibility to malaria, *Aotus lemurinus griseimembra* currently represents the best malaria primate model because of its high susceptibility to infection by blood forms and sporozoites of both species of *Plasmodium*. *Aotus* also represents a good model for gaining insight into the pathogenesis of severe anemia,¹⁵⁰ screening of antimalarial drugs, and the understanding of malaria pathogenesis as well.⁹⁴ In view of the decreasing availability of these primates, breeding programs in biomedical research facilities must be improved.⁹⁴

Plasmodium malariae and *P. ovale* have been shown to experimentally develop in splenectomized New World *Aotus* (*Aotus azare boliviensis* and *A. lemurinus griseimembra*), as well as *Saimiri sciureus boliviensis* monkeys and chimpanzees (*Pan troglodytes*). The establishment of *P. malariae* and *P. ovale* isolates in these animals has aided the development of diagnostic probes and the identification of areas of antigenic variation within the species and understanding the parasitology of the parasites.¹⁵¹

Primate models have been used to establish clinical parameters of malaria infection. In one study,¹⁵² three methods for the quantitation of parasitemia in malaria were compared with the standard method in *Plasmodium berghei*–infected mice and *P. knowlesi*–infected rhesus monkeys. The technique, in which parasitemia was calculated from the number of peripheral red blood cells (PRBCs) per oil immersion field (OIF) and the estimated amount of blood in one OIF of a thick smear, was most accurate when parasitaemia was low (<1%) in both malaria patients and experimental animals. Thus, this technique, which is simple and cost effective, is used routinely for quantitation of parasitemia.

The availability of both natural and artificial hosts combined with the close phylogenetic relationship between nonhuman primates and humans make simian malaria parasite infection in nonhuman primates attractive to study host–parasite interaction in detail.¹⁰¹ *Plasmodium knowlesi*, a highly pathogenic simian malaria parasite of macaques (*Macaca fascicularis* [natural host] and *M. mulatta* [experimental host]) has been used to study several aspects of malarial infection.¹⁰⁴ Baboons (*Papio anubis*) experimentally infected with *Plasmodium knowlesi* developed both acute and chronic infections, and subsequent reinfection was established in previously infected and treated animals. These results show that a baboon *P. knowlesi* model offers an advantageous alternative to macaques in that baboons are capable of showing both acute and chronic disease progression, combining the characteristics of *P. knowlesi* infection of *M. fascicularis* (chronic) and *M. mulatta* (acute) in a single model.¹⁰¹

The pathogenesis of human cerebral malaria is suspected to be caused by blockage of cerebral microvessels by the sequestration of parasitized human red blood cells (PHRBC). Examination of

infected tissues indicate PHRBC sequestration in microvessels is the result of PHRBC knob attachment to endothelial cell surface cytoadherence receptors such as CD36, thrombospondin (TSP), and intercellular adhesion molecule-1 (ICAM-1). In lieu of fresh human tissue, several animal models for human cerebral malaria have been developed; the *Plasmodium coatneyi*-infected rhesus monkey model is the most versatile.^{153,154}

Immunology

The role of T cells in pathogenic infections including malaria has been investigated using the *Aotus* monkey model.¹⁵⁵ Gamma-delta T cells are implicated to play crucial roles during early immune responses to pathogens. However, the precise role of these cells and the ligands recognized in human immune responses against pathogens remains unclear because of the lack of suitable animal models. A study by Daubenberger and colleagues¹⁵⁵ analyzed the reactivity of spleen cells of the New World monkey *Aotus nancymae* against metabolites selectively activating gamma-delta T cells. The structural and functional conservation of gamma-delta T cells in *A. nancymae* and humans implicated a functionally important and evolutionarily conserved mechanism of recognition of such pathogen metabolites. *Aotus* monkeys have also been used in studies of T lymphocyte-specific antibodies. Billie et al.¹⁵⁶ have also shown that *Plasmodium coatneyi* in the rhesus monkey (*Macaca mulatta*) is a suitable model for studies of malaria in pregnancy.

Vaccination

Although the ultimate validation of the usefulness of monkey model depends upon human vaccine trials, over the past two decades monkeys have proven very useful in testing multiple malaria vaccine candidates prior to trials in humans. A good correlation between the B- and T-cell epitopes recognized by humans and by immunized *Aotus* monkeys has been documented, and cross-reactivity between reagents for human and *Aotus* cytokines and lymphocyte markers has been identified and is facilitating the selection of vaccine candidates for clinical trials. Synthetic peptide and recombinant protein vaccines are optimally immunogenic when delivered with an effective adjuvant. Such studies have been carried out in the *Aotus* monkey and other nonhuman primates. Candidate vaccines currently insufficiently immunogenic might induce a protective immunity if they could be delivered with more effective adjuvants. For example, immunogens that induce promising responses when administered to mice with complete and incomplete Freund's adjuvants perform less well in primate animal models where a complete Freund's adjuvant is not used. The use of synthetic oligodeoxynucleotides containing CpG motifs, the sequences of which are based on immunostimulatory bacterial DNA sequences, to enhance the immune response in *Aotus* monkeys to a synthetic peptide malaria vaccine has been reported.¹⁵⁷ These data indicate that oligodeoxynucleotides containing CpG motifs improve immunogenicity of peptide immunogens in non-human primates and may be immunopotentiators useful in humans.

The rhesus macaque model is important in preclinical vaccine development.¹⁰⁴ These South American monkeys have been used as animal models to assess the immunogenicity and protective efficacy of the *P. falciparum* DNA vaccine using cholera B toxin as an adjuvant.¹⁵⁸ *Plasmodium knowlesi* malaria in rhesus macaques provides a nonhuman primate model for malaria vaccine development allowing reliable, stringent sporozoite challenge, and evaluation of both cellular and antibody responses is needed.¹⁰⁴ The efficacy of the ant sporozoite peptide malaria vaccine combined with copolymer-based adjuvants has also been evaluated in rhesus monkeys.¹⁵⁹ Immunogenicity and efficacy tests with compounds representing parts of the 75 kDa merozoite surface antigen of *P. falciparum* have also been tested in *Aotus nancymai* monkeys,¹⁶⁰ and a recombinant vaccine expressed in the milk of transgenic mice protects *Aotus* monkeys from lethal challenge with *P. falciparum*.¹⁶¹

Other studies have focused on Pf72/Hsp70-1 antigen, a major target in naturally acquired immunity against *P. falciparum* malaria.¹⁶² Significant differences were revealed in the panel of B-cell epitopes on this molecule recognized by animal models including primates and by humans sensitized by natural infection. The results suggested that the multiple antigenic peptides (MAP) strategy is particularly useful as a means of obtaining suitable synthetic models for conformation-dependent epitopes for use in vaccination.

Table 9.2 summarizes the animal models for the study of malaria.

ONCHOCERCIASIS

Onchocerciasis is a chronic filarial disease caused by *Onchocerca volvulus*, a nematode transmitted by black flies of *Simulium* species, of which the most common is *S. damnosum*. Adult and microfilarial stages of the parasite affect mainly the skin, eyes, and lymph nodes. Heavy infection leads to general debility, chronic puritis, disfiguring skin lesions, visual impairment, and blindness. Clinical features and pathology are caused mainly by inflammatory reactions around damaged and dead microfilariae.¹⁶³ Disease variations are due to differences in parasitic strains, degree and frequency of infection, and host differences, which include nutritional state and immune responses to parasitic antigens. According to Cheesbrough,¹⁶⁴ the main clinical features include formation of nodules and dermatitis and inflammatory reactions in the eye, which may lead to blindness. Onchocerciasis is thought to be a risk factor for epilepsy.

A total of 35 countries are affected in total, 28 in tropical Africa where 99% of infected people live. Isolated foci in Latin America countries (six countries) and Yemen (<http://www.who.int/tdr/diseases/oncho/diseaseinfo.htm>) have also been reported.

Etiology, Transmission, and Life Cycle

Onchocerca volvulus adults are long and threadlike. They are white/cream with blunt tapering extremities. The female measures 230 to over 500 mm × 0.25 to 0.50 mm, while the male is much smaller, measuring 19 to 42 mm × 0.23 to 0.15 mm.¹⁶⁵ Adult worms lie coiled or tangled within subcutaneous nodules or are found wandering in the subcutaneous tissue en route to dermal sites. Nonperiodic microfilariae are found in the intercellular spaces and in the chambers of the eye.

Table 9.2 Animal Models for Human Malaria

Experimental Animal Model	Human Malaria Parasite	Aspect of Malaria Study
Aotus monkeys	<i>P. falciparum</i> , <i>P. vivax</i>	Clinical, immunology, vaccine, drugs, parasite molecular biology, anemia
Rhesus macaque monkeys	<i>P. falciparum</i>	Pregnacy, clinical, immunology, vaccine and drugs
Chimpanzees (splenectomized)	<i>P. falciparum</i> , <i>P. malariae</i> , <i>P. vivax</i> , and <i>P. ovale</i>	Parasitology and immunity
Squirrel monkeys	<i>P. falciparum</i>	Clinical, parasitological and haematological
Transgenic mice	<i>P. falciparum</i>	Parasitological and parasite biology

They have also been found in urine and rarely in the blood. Dead microfilariae, lying straight, have been found in the cornea. Their morphology can be studied after staining with Giemsa or in section with hematoxylin and eosin. Unsheathed microfilariae have rounded heads and sharply pointed tails and measure $300 \text{ to } 360 \times 5 \text{ to } 9 \mu\text{m}$.

Transmission is through a bite of the appropriate species of the black fly. Person-to-person infection does not occur. Congenital transmission rarely occurs from heavily infected mothers to infants. Black flies feed on a wide range of animal species. Natural infections have been reported in the gorilla (*Gorilla gorilla*) in Africa and the spider monkey (*Ateles geoffroyi*) in the new world tropics, but the human is the only important known host of *O. volvulus*.

Simulium black flies are the obligate intermediate hosts of *O. volvulus*. The flies breed in fast-flowing rivers, and infective-stage larvae (L3) are released from infected black flies during a blood meal. L3 larvae undergo two molts to become adult worms that pass into the interstitial fluids of skin, eyes, and other organs. The development of infective larvae to adults takes 2 to 3 months, and microfilariae appear a year or more after infection. Microfilariae live for 6 to 30 months, while the adult worms live for 10 to 15 years and can be detected in subcutaneous collagenous nodules.

Females release approximately 1000 microfilariae (L1) per day over a 9- to 14-year period, and the cycle is continued after uptake by black flies during a blood meal. The standard diagnostic test for onchocerciasis is the skin snip, performed by placing a small piece of skin in a drop of saline or culture medium.¹⁶⁴ After incubation, microscopic examination reveals microfilariae emerging from the tissue. Despite the presence of a heavy worm burden, the skin of most infected individuals appears normal, and histological examination shows parasites in the dermis with no inflammatory response.

Clinical Manifestations

Clinical manifestations are thought to occur when the parasites die and initiate an inflammatory response. It is the host response to the parasites, rather than direct cytotoxic molecules released from the parasite, that is generally thought to be responsible for disease. This notion is supported by studies in which individuals treated with the anthelmintic diethylcarbamazine develop severe puritis, presumably as a result of rapid death of microfilariae in the skin.¹⁶⁶

Most of the tissue damage in onchocerciasis is in the skin or eyes and is caused by dead or dying microfilariae. They induce the host fibrous response that encapsulates the worms. Clinical expression varies widely between geographic areas as well as between individuals in the same area, due to the sites of vector bites and differences in the vector strains. Three distinct clinical manifestations can be described.

Lymph Node Enlargement

Generalized superficial lymph node enlargement may be seen. In Africa, most prominent nodes appear in the femoral triangle and groin, often firm to hard and slightly or moderately enlarged. Gross enlargement of inguinal and femoral nodes accompanied by locally atrophic skin predisposes to the development of "hanging groin."

Nodule Formation

These nodules may also be referred to as onchocercomas. They form under the skin when adult worms are encapsulated in subcutaneous tissue. The nodules are smooth, firm, and rubbery and may be round or elongated, measuring 5 mm across up to 50 mm when found in clusters. In many parts of Africa, nodules are found on the lower part of the body, around the pelvis. In Central America and the savanna areas of Africa, nodules form on the upper parts of the body, while in

young children below the age of 9 years, nodules form mainly on the head. In Yemen, mainly the lower limbs are affected. Inflammatory dermatitis is accompanied by intense irritation, raised papules on the skin, and alteration in skin pigment.

Blindness

Microfilariae may also migrate to the eyes. In early infections, microfilariae are found in the cornea and the anterior chamber, which causes redness.¹⁶⁴ Progressive changes occur, leading to sclerosing keratitis and later to blindness. The iris can also be infected, while inflammation of the choroids and the retina also lead to blindness.

Sowda

“Sowda” (black disease) is used to describe severe allergic responses usually affecting only one limb, with darkening of the skin. Lymph nodes draining the limb become swollen and painful. According to Miller and co-workers¹⁶³ in chronic cases, the skin loses elasticity and becomes wrinkled, making people appear aged (elephant skin). When the skin around the groin is affected, “hanging groin” develops, while “leopard skin” refers to a spotted depigmentation of the skin associated with chronic cases. Sowda is accompanied by severe itching, and the skin becomes dark and often edematous. It may also be accompanied by a rash. Microfilariae are few and restricted to the areas of dermatitis.

Animal Models

Although *O. volvulus* infects chimpanzees, gorillas, and cynomolgus monkeys, no animal model of natural infection with *O. volvulus* results in ocular disease. Experimental animal models have therefore been developed that reproduce many of the clinical manifestations observed in human disease.

Guinea Pigs

Donnelly and collaborators¹⁶⁷ showed that guinea pigs develop ocular disease characterized by limbitis, corneal inflammation, and peripheral corneal neovascularization after intracorneal inoculation of the cattle parasite *Onchocerca lienalis*. They demonstrated that the inflammatory response and the severity of keratitis are greatly exacerbated if the animals are sensitized prior to ocular challenge, thereby implying a role for specific immunity. They also noted that diethylcarbamazine-induced killing of the parasites results in an exacerbated inflammatory response, which is consistent with the notion that the pathology associated with keratitis is due to a host immune system-mediated inflammatory response. Clinical manifestations similar to onchocercal keratitis can be induced in guinea pigs by direct injection of soluble *O. volvulus* antigens into the corneal stroma if the animals have been previously sensitized.

Rabbits

The development of a protective immune response to *O. volvulus* infection must involve parasite antigens, which are accessible to the host immune system. In an attempt to characterize such antigens present in *O. volvulus* infective larvae (L3), an antiserum was produced by injection of viable L3 into a rabbit, and then the antiserum was used to screen a cDNA library prepared from adult stage mRNA and several cDNA clones identified, which reacted with the antiserum.¹⁶⁸

Chandrashekar and collaborators¹⁶⁹ reported that sera from humans infected with *O. volvulus* contain parasite antigens in immune complexes that are detectable by immunoblot with polyclonal rabbit anti-*O. volvulus* antiserum. Rabbit anti-*O. volvulus* antibodies were produced by repeated intramuscular injections of *O. volvulus* adult worm crude antigens in complete Freund's adjuvant.

Mice

Mice have been used in hybridoma production. Mice were immunized by subcutaneous injection of 100 µg of *O. volvulus* antigen (OvAg) depleted of phosphorylcholine (PC) components in complete Freund's adjuvant on days 1 and 29.¹⁶⁹ Serum collected on day 36 was screened for OvAg by enzyme-linked immunosorbent assay (ELISA) and to circulating antigens in polyethylglycol precipitates by immunoblot. Two mice chosen for fusion were boosted with 75 µg of OvAg depleted of PC by incomplete Freund's adjuvant by subcutaneous injection 4 days prior to fusion.

Monoclonal antibodies to circulating *O. volvulus* antigens have also been produced and characterized using mice.¹⁶⁹ Two monoclonal antibodies (OV-1 and OV-5) that bound to circulating *O. volvulus* antigens were identified; both isotypic as IgG_{2b} antibodies, and they bound to the same circulating parasite antigens (23, 28, 50, 54-58, 62, and 70 kDa) immunoblots of polyethylene glycol PEG-precipitated immune complexes from human onchocerciasis sera that were recognized by rabbit anti-*O. volvulus* antibodies.

In another experiment, it was demonstrated that prior immunization is necessary for the induction of pathology in mice. In addition, studies have demonstrated that T cells are required for the development of keratitis, since athymic mice that were immunized and challenged intrastromally with *O. volvulus* antigen did not develop keratitis.¹⁷⁰ Furthermore, adoptive transfer of spleen cells from immunized but not from naive mice reconstitute the development of keratitis in these animals after intrastromal challenge. Although these studies did not identify specific subpopulations of cells, mRNA for CD4 and CD8 cells was detected in recipient corneas. As observed during human infections, mice develop a predominant Th2 response to repeated exposure to *O. volvulus* antigen; *O. volvulus*-stimulated lymph node and spleen cells from immunized mice produce IL-4 and IL-5 and little IFN-γ. This response is also manifested locally, since mRNA for IL-4 and IL-5 is upregulated in the corneas of immunized mice upon intrastromal challenge.¹⁷⁰

This indicates that the severity of keratitis is regulated by these cytokines and that the regulation is associated with infiltration of inflammatory cells, notably eosinophils, into the cornea. The association between disease severity and presence of a Th2 response suggested that modulation of the Th2 response would reduce the severity of keratitis. Therefore, two approaches were taken:

1. Ablation of the Th2 development by using IL-4 gene knockout mice
2. Induction of a Th1 response by using recombinant IL-12

Other Animal Models

Experimental infections of chimpanzees with *O. volvulus* and cattle with *O. ochengi* have been used to provide models for research in human onchocerciasis.¹⁷¹ The antibody responses of cattle infected with *O. ochengi* to 18 recombinant *O. volvulus* antigens were measured by ELISA. Cynomolgus monkeys have also been used in experimental ocular onchocerciasis elicited by *O. volvulus*.¹⁷² Infection of cynomolgus monkeys with *O. lienalis* has also been studied as a model for human onchocerciasis.¹⁷³ Mangabey monkeys and patas monkeys have also been used.¹⁷⁴ Sporadic cases of ocular *Onchocerca* sp. infection have been reported in dogs in the U.S. and Europe.¹⁷⁵ These ocular onchocerciasis infections in dogs may provide a useful experimental system for human onchocercosis. Adults of *Onchocerca* sp. infecting dogs, *O. volvulus* of humans, and *O. lienalis* infecting cattle were found to be similar, with only moderate morphological differences; nevertheless, the morphology of microfilaria of *Onchocerca* in dogs is unique within the genus.¹⁷⁶ Recent

studies on isolates causing canine subconjunctival infection in Greece and Hungary demonstrated that the same *Onchocerca* species was responsible for the infection in the two countries.¹⁷⁷

Comparison of the Different Experimental Animal Models

Experimental infections of chimpanzees with *O. volvulus* and cattle with *O. ochengi* have provided model systems for research in human onchocerciasis. To compare the two models, recombinant antigens of *O. volvulus* (Ov7, Ov103, and B20) were used to analyze the kinetics of antibody production following experimental infection.¹⁷¹ Both chimpanzees and cattle responded to Ov7 and Ov103, but only 33% of the chimpanzees responded to B20. Responses, however, showed similarities in the two models, in that responses to Ov7 and Ov103 peaked after the onset of patent infections while responses to B20 peaked before. Onchocercal infection in cattle with *Onchocerca gibsoni* has been used extensively as an animal screening system for chemotherapeutic agents. Its advantage is the presence of nodules formed, similar to those observed in *O. volvulus* infection in humans.¹⁷⁸ Mangabey monkeys have been shown to support complete development of the parasite.

In guinea pigs, the inflammatory response and the severity of keratitis are greatly exacerbated if the animals are sensitized prior to ocular challenge, thereby implying a role for specific immunity. Killing of parasites, e.g., by use of diethylcarbamazine, results in an exacerbated inflammatory response.¹⁶⁷ Natural infections have been reported in the gorilla (*Gorilla gorilla*) in Africa and the spider monkey (*Ateles geoffroyi*) in the new world tropics. Although *O. volvulus* infects chimpanzees, gorillas, and cynomolgus monkeys, there is no animal model of natural infection with *O. volvulus* that results in ocular disease.

Mice have been the most widely used models for onchocerciasis. They can be induced to kill larvae through immune-mediated processes, for example by injecting them with freeze-thawed *Onchocerca* species. Both eosinophils and IL-5 have been found to increase consistently with the time of parasite killing. Elimination of IL-5 and IL-4 has been found to reduce the protective effects of vaccination against *O. volvulus*.¹⁷⁰ This has therefore made the mice a good model for vaccine development and also for the study of the pathogenesis of onchocerciasis.

Immune Responses

Antibodies can be detected by a variety of immunodiagnostic techniques, though they are not protective and no correlation exists between antibody levels and disease severity. Nonspecific IgG and IgE concentrations in the circulation are higher in patients with onchocerciasis.¹⁷⁹ IgE serum levels in children are found to be related to microfilarial loads to some extent. Ikeda and coworkers,¹⁸⁰ using indirect hemagglutination, showed a positive correlation between antibody titers and microfilarial density. Kawabata and collaborators,¹⁸¹ using ELISA, found that specific IgG, IgM, IgA, and IgE antibodies to *O. volvulus* antigens are present in higher concentrations in people infected with onchocerciasis. It was also found that patients with <5.0 microfilariae/mm² of skin in the skin biopsies had significantly lower levels of specific IgM antibodies.

The immune response in onchocerciasis is mainly cell mediated. Impairment of the cell-mediated immune response is very common and widespread in onchocerciasis endemic areas and has been associated with exposure to the antigens before the age of immune competence. Eosinophils are the predominant inflammatory cells in the cornea after injection of soluble parasite antigens into the corneal stroma, and there is good evidence to implicate eosinophils in pathogenesis.¹⁸² The severity of keratitis observed in the experiments correlated with the number of eosinophils in the cornea. For example, IL-4 gene knockout mice developed less severe keratitis than did control animals, and significantly fewer eosinophils were present in the corneas of the knockout mice. Conversely, systemic administration of recombinant IL-12 resulted in exacerbated keratitis, which was associated with increased numbers of eosinophils in the cornea.

Although eosinophils are strongly associated with the development of keratitis, recent studies indicate that neutrophils can also mediate onchocercal keratitis.¹⁸³ Temporal analysis of the cellular response to *O. volvulus* antigens indicated that although eosinophils were the most prominent inflammatory cells at the time of maximal keratitis, there was also an infiltrate of neutrophils that peaked at an earlier time point. Mice deficient in IL-5 (which is essential for the development and maturation of eosinophils) still developed corneal opacification comparable in severity to that in immunocompetent mice.

The observation that a minority of individuals in areas of endemic infection have no sign of infection provides circumstantial evidence for naturally acquired resistance. Given these findings, a number of studies have examined the immune responses underlying acquired resistance to infective-stage larvae and microfilariae. In a model in which resistance to infective-stage larvae (L3) is induced by immunization with irradiated parasites, protection is dependent on IL-4 and IL-5, and eosinophils are associated with degenerating parasites. An essential role for IL-4 in the protective response to L3 larvae was further supported by a recent study with IL-4 gene knockout mice. Similarly, acquired resistance to *O. lienalis* microfilariae is IL-5 and eosinophil dependent. However, IL-4 is not required for the development of resistance to the microfilarial stage, since IL-4 gene knockout mice continue to produce IL-5, and resistance in these animals remains IL-5 dependent. It has been suggested that induction of resistance, but not immunopathology, may depend on being able to stimulate IL-5 without stimulating IL-4.¹⁸⁴ Taken together with observations that some regions of individual *O. volvulus* proteins, such as protein disulfide isomerase, are more likely to induce keratitis than others are, further directions aimed at vaccine development without pathogenesis may require manipulation of both the host immune response and parasite proteins.

Immunosuppression has also been used to explain the presence of microfilariae in tissues free of inflammation. Immunosuppression by factors from *O. volvulus* has been described involving proteins of a molecular weight similar to albumin¹⁸⁵ and a low molecular weight (<10,000). Greene¹⁸⁶ suggested that the soluble factors are involved in modulating lymphocyte function in human onchocerciasis.

Peripheral blood mononuclear cells from patients with generalized microfiladermia and no clinical symptoms have a suppressed proliferative response to parasite antigens. Such individuals also produce cytokines associated with a T helper type 2 response compared with individuals exposed for a short time. Consistent with this finding, Limaye and collaborators¹⁸⁷ found elevated IL-5 levels in the serum of patients treated with diethylcarbamazine, which rapidly kills microfilariae and induces eosinophilia. However, putatively immune individuals, who are resident in areas of highly endemic infection but have no indication of infection, also have elevated IL-2 and IFN- responses.

In relation to disease manifestations, Freedman and coworkers¹⁸⁸ compared peripheral blood leukocyte responses from individuals with and without ocular manifestations. They reported that *in vitro* stimulation with parasite antigens of cells from individuals with ocular onchocerciasis produced more IL-4, IL-5, and IL-10 than did stimulation of cells from infected individuals with no ocular disease. These observations imply that ocular pathology is associated with elevated Th2 responses.

SCHISTOSOMIASIS

Introduction

Schistosomiasis disease is endemic in 76 countries in the tropics, with more than 80% of infected people living in sub-Saharan Africa.^{189,190} The disease continues to spread in the face of sustained control measures and availability of an effective drug, praziquantel. The number of infected people is estimated to be 250 million, while the number at risk of infection is about 600

million.^{191,192} Of those infected, the number of symptomatic patients is 120 million, while 20 million are severely affected. The disease burden has been calculated to be 1.93 million Disability Adjusted Life Years (DALY).¹⁹³ This figure is thought to be a gross underestimation, and the burden of disease has recently been reassessed, suggesting that approximately 70 million individuals suffer from haematuria associated with *Schistosoma haematobium* and 18 million suffer from major bladder wall pathology. Annual mortality due to *S. haematobium*-related nonfunctioning kidneys could be as high as 150,000. Morbidity due to *Schistosoma mansoni* includes hepatosplenomegaly, liver fibrosis, and ascites, and as many as 130,000 people die each year from hematemesis and related portal hypertension. The host granulomatous response to eggs in intestines or those translocated to the liver or lungs results in significant morbidity, and in highly endemic areas, where between 60 and 80% of individuals may be infected, 5 to 10% of infected individuals exhibit clinical illness due to the resulting pathology. Evidence from Sudan and Uganda suggests that in most cases serious liver fibrosis takes more than 15 years to develop.^{194,195} Children are especially vulnerable to schistosomiasis, and infected school-aged children are often physically and intellectually compromised by concurrent anemia, attention deficits, learning disabilities, school absenteeism, and higher dropout rates.^{192,196} In rural areas of many developing countries, schistosomiasis is an important occupational hazard.¹⁹⁷ It has also been clearly shown to have a significant impact on overt pathology and on diffuse parameters such as nutrition, growth, and physical well-being,¹⁹⁸ including areas such as cognition that are difficult to assess. Thus the socioeconomic impact and public health importance of this parasitic disease in endemic areas is surpassed only by that of malaria.

Life Cycle and Parasite Biology

The genus *Schistosoma* belongs to the subclass Digenea. The Digenea undergo an alternation of generations and of hosts. Adult Digenea live in the veins of vertebrates, while the intermediate hosts are mollusks. In the case of schistosomes, fresh-water snails act as intermediate hosts. The schistosomes are equipped with suckers, with which they can attach to the blood vessels of the host. Unlike all other Digenea, which are hermaphroditic, schistosomes are dioecious. The three major schistosome species that infect humans are *S. haematobium*, *S. mansoni*, and *S. japonicum*, but others such as *S. intercalatum* and *S. mekongi* also infect humans to a much lesser extent. On rare occasions, humans may also be parasitized by animal schistosomes such as *S. bovis*, *S. curassoni*, *S. margrebowiei*, *S. mattheei*, and *S. rodhiani*.

Schistosomes have a complex life cycle. The adult worms do not multiply in the vertebrate host. A portion of the approximately 300 eggs excreted by the mature female worm per day will reach an invertebrate host in which cycles of asexual reproduction occur. Eggs that have not been lodged in body organs of the host are primarily excreted in the urine (*S. haematobium*) or feces (*S. mansoni* and other species). If the eggs come into contact with fresh water, a larva known as a miracidium hatches. To continue the life cycle, the miracidium must enter an appropriate host, which for *S. mansoni* is snails of the *Biomphalaria* genus. In the snail, several generations of asexually dividing larvae, sporocysts, develop. These eventually produce large numbers of infective larvae, the cercariae, which emerge out of the host under appropriate photostatic conditions.¹⁹⁹ This procedure of stimulation of cercarial shedding by light exposure is exploited in the laboratory situation, when parasites are to be collected from patently infected snails. Up to 3000 cercariae/snail/day may be released. These are able to penetrate the skin of the final host. During penetration, the tails are shed, and the cercariae enter the bloodstream as motile immature schistosomula. The schistosomula migrate to the lungs in 3 to 8 days and then to the liver, where the adult worms pair, approximately 26 days after infection. The paired adult worms migrate against the venous blood flow of the vena aorta to the predilectic sites, which in the case of *S. mansoni* are the mesenteric veins. It is here that egg production starts about 40 days after infection. In humans, the number of worms per infected individual is estimated to be fewer than 20, although

in extreme cases as many as 2000 worms have been encountered.²⁰⁰ Distribution of schistosomes in endemic populations is described as being overdispersed, i.e., many individuals harbor only light infections, while a few have massive parasite loads. The lifespan of adult schistosomes is typically 3 to 7 years, although survival of more than 30 years has been reported.^{199–201} Of the three main species, *S. mansoni* has proven to be the most easily maintained in the laboratory and lends itself easily to experimental infection of mice and primates. This has made *S. mansoni* the subject of most research attention.

Animal Models

Many animals have been found naturally infected, possibly representing an important zoonotic aspect of schistosomiasis transmission. These species permit studies of the basic life cycle, pathology, immunology, and chemotherapy. Rodents, especially mice and rats, are the most widely used. Marked differences occur in response to infection between species and even between strains within species.²⁰² Thus many rat strains throw off primary infections and develop either a temporary or permanent resistance to reinfection. Most mouse strains retain the primary infection and develop only a partial resistance to reinfection; mouse infection produces severe pathology. The relevance of immune responses of inbred mouse strains to humans is open to question, and hence there is a need to carry out studies in primate models. Another constraint is that the average lifespan of schistosomes is substantially longer than that of a mouse, effectively precluding long-term studies in this model. Moreover, the metabolism of mice differs from that of humans, so that schistosomicidal drugs effective in humans may show little activity in rodent models. The following sections summarize the salient features of schistosomiasis studies in rodent and primate models.

Mice

Experimental investigations of immune responses and pathology commonly employ rodent models such as mice or rats infected with *S. mansoni*. Implications of Th1 vs. Th2 responses were mainly described in mouse models, since both molecular and cellular immunology studies were facilitated by the availability of immunological tools and the production of transgenic and knockout mice. In this model, it has been shown that a Th2 response was involved in the development of lesions.²⁰³ These findings are in contrast with those observed previously in humans and more recently in primates.²⁰⁴ Indeed, the only immune correlate of protection in baboons, as in humans, was levels of specific IgE antibodies, supporting a beneficial role of a Th2 response. Taken together, these observations provide a cautionary note on extrapolations of results obtained in the mouse model to other species and underline the need for multiple species analysis of the immune response.

Mice have been used to study coinfections including that between schistosomiasis and murine acquired immune deficiency syndrome (AIDS).²⁰⁵ This study explored the controversial concept that a Th2 environment as occasioned by parasite infection could be associated with disease progression, raising the possibility that helminth infections might accelerate retroviral disease progression. This study demonstrated that infection of mice with *S. mansoni* neither enhanced Th2 cytokine production nor accelerated murine AIDS progression in animals subsequently challenged with a retroviral complex.

Rats

Several studies in a semipermissive host of schistosomiasis, the rat model, have implicated different immune mechanisms, including anaphylactic antibodies and nonlymphoid cells in rejection of worms between 3 and 4 weeks after a primary infection and in the development of immunity to reinfection.²⁰⁶ Effector mechanisms against *S. mansoni* have shown that antibodies are cytotoxic for schistosomula in the presence of effector cells, including eosinophils, macrophages,

and platelets. In the rat model of schistosomiasis, humoral immunity seems to play a critical role in the mechanisms of defense; however, cell-mediated responses are insignificant. This model allowed the identification in detail of the isotypes involved in the antibody-dependent cell-mediated cytotoxicity.^{207,208} These results suggested that blocking antibodies could be considered as a new mechanism regulating immunity in parasite infection. In murine schistosomiasis, although protection can be offered by specific antibody transfer, the role of humoral immunity during experimental infection is negligible. There is a striking similarity in antibody responses including isotype regulatory networks in rats and humans that confirms the usefulness of the rat model in our understanding of humoral responses during schistosomiasis.

The rat model has shown that Th2 cytokines (IL-4 and IL-5) appear to be associated with resistance since they control the production of IgE and eosinophilia.^{209,210} These findings are in contrast with those obtained in the murine model, in which Th2 responses have been associated with pathology.^{211,212} Both the rat and mouse models have served to enhance understanding of pathogenetic mechanisms of liver fibrosis during schistosomiasis disease. There have been important common findings derived from several different models that established the involvement of Ito cells and TGF-B in liver fibrogenesis.²¹³

Nile Rats

Reports of domestic and wild animals that are naturally infected with *Schistosoma* spp. have included the Nile rat (*Arvicanthus niloticus*). One study from the Gezira irrigation scheme in Sudan reported infection rates of 4.9% in Nile rats.²¹⁴ A natural double infection of *S. mansoni* and *S. haematobium* was also reported from a human endemic area in Egypt.²¹⁵ These studies concluded that Nile rats present in large numbers and often found in or near canals may be involved in schistosome transmission and could act as reservoir hosts. Experimental infections have also been reported,²¹⁶ but no IgE was found in the serum of infected Nile rats.²¹⁷

Hamsters

Hamsters (*Mesocricetus auratus*) have been used extensively as models of schistosomiasis. The susceptibility of hamsters to *S. mansoni* infection compares very well with that in other animal models such as rats, mice, and monkeys.²¹⁸ A comparison of the four animal models showed the same linear relationship between total worm burdens and the proportion of male adult *S. mansoni* worms.²¹⁸ Early studies also looked at the comparative efficiency of different regimes for infecting laboratory rodents in order to find out optimal conditions under which *S. mansoni* infections can be maintained.²¹⁹ Analysis of the data showed that for mice, infection with 150 cercariae was optimal, while for hamsters 200 cercariae were required, and both golden and hairy-footed hamsters were shown to be equally susceptible to *S. mansoni* infection.²¹⁹ Both intravenous and subcutaneous routes of infection produced high rates of infection in golden hamsters, while the same routes produced lower levels of infection in cotton rats.^{219,220}

Studies of coinfection involving leishmania and schistosome parasites have been performed in golden hamsters. Other coinfection studies have looked at the association of salmonellosis and schistosomiasis using the hamster model.^{221,222} These studies showed that a direct physical relationship between bacteria and worms facilitates establishment of *Salmonella paratyphi* A *in vivo* and that a deficit in host immune response is normally a factor involved in enhanced growth of salmonella.²²¹ A hamster model of schistosomiasis provided the first opportunity to examine the early phases of the development of portal hypertension in a rodent model of chronic liver disease.²²³ Although there is marked variability in development of renal lesions in individual animal models of schistosomal infections, much has been learned from studies in hamsters and other rodents.²²⁴ Renal pathology in *Schistosoma haematobium* infections of hamsters was related to the lower urinary tract, with obstructive lesions causing pyelonephritis and hydronephrosis.²²⁴ The relationship

between parasite infestation and chemical mutagen metabolism was investigated using *S. haematobium* infection in hamsters.²²⁵ Hamsters have also been used alongside mouse models to examine the relationship between host leucocyte reactions to schistosomula and innate or acquired resistance to *S. mansoni*.²²⁶ Studies of schistosomiasis chemotherapy have also utilized the hamster model. The ability of carnosine to improve some liver disorders induced by *S. mansoni* parasites was studied in hamsters.²²⁷ It was shown that carnosine has an effect on procollagen III peptide levels, which were lowered in infected hamsters treated with carnosine.²²⁷

Gerbils

Gerbils (*Meriones unguiculatus*) have been used as models for studies of cercarial dermatitis, which occurs when humans are infested with avian bilharzias species. Investigations showed that once the skin barrier has been overcome, the schistosomula migrated into the lungs of the host, where they survived and induced lesions.²²⁸ In gerbils, these lesions persisted for weeks after exposure but worms were not identified in the mesenteric vessels.²²⁸ The presence and development of adult worms in the lungs of gerbils infected with *S. mansoni* have also been studied.²²⁹ These studies revealed that many eggs embolized in the lungs of gerbils and a few in brain vessels.

Although glomerular lesions frequently occur in schistosomiasis patients, appropriate animal models for the study of schistosomal glomerulonephritis have not been developed. A recent study used gerbils to analyze the relationship between infection levels and glomerulonephritis.²³⁰ The findings suggested that T cells might be involved in the development of glomerulonephritis, and gerbils could be a useful model to clarify the role of such T cells. Gerbils have also been used in vaccination experiments against schistosomiasis. In gerbils, the attenuated schistosome vaccine is known to induce marginal or no resistance to a homologous infection.²³¹ Recent studies have postulated that in gerbils limited recruitment of dendritic cells around attenuated larvae at least partially contribute to defective induction of protective immunity by attenuated larvae.^{232,233} A study that made a comparison of new models of schistosomiasis such as the marsh rat (*Holochilus brasiliensis*), jirds (*Meriones shawi*), and gerbils against classical models such as mice concluded that the new models are perfectly utilizable in laboratory settings.²³⁴ The models proved helpful in investigating the pathogenic mechanism of some disorders in *S. haematobium* infection, particularly tumors of the urinary bladder.²³⁴

Guinea Pigs

The guinea pig has been studied as a potential laboratory host for *S. mansoni*, as reviewed by Pearce and McLaren.²³⁵ Twenty-six percent of an infective cercarial dose survived to maturity in this rodent model, and no gross fluctuations in worm burden subsequent to pairing of male and female parasites occurred. Schistosomes that mature in guinea pigs have topographical features identical to those exhibited by similarly aged mouse worms but different from those exhibited by rat worms. Schistosome eggs are never detected in the feces of infected guinea pigs but can be observed in pulmonary, hepatic, and intestinal tissues. Only 55% of the eggs that can be recovered from intestinal tissue are viable and can be hatched to release miracidia that penetrate the intermediate snail host. Cercariae are sometimes liberated from infected snails but in insufficient numbers to permit infection of naïve guinea pigs. The schistosome cycle cannot therefore be completed in this host. Collaterals are sometimes observed in the vasculature serving the rectum and kidneys of infected guinea pigs, and the fact that schistosome eggs are deposited in the lungs of these animals indicates that portal systemic anastomosis is a feature of the model. Pathology associated with cercarial invasion or egg deposition is not dissimilar to that described for other laboratory animals infected with *S. mansoni* except that basophils participate in the inflammatory response observed in the skin and intestine.

In vivo and *in vitro* parameters of immunity have been assessed in guinea pigs exposed to 500 normal or 500 radiation-attenuated cercariae of *S. mansoni*.²³⁶ High levels of resistance to a challenge infection developed in both the chronic and irradiated vaccine models, but immunity was expressed earlier (week 4) and reached higher levels (90%) in the vaccine model. Vaccinated guinea pigs have thus been shown to achieve greater resistance than more commonly used rodent hosts. *In vitro* cytotoxicity assays demonstrated that antibodies capable of participating in complement-dependent (lethal antibody) or eosinophil-mediated schistosomular killing develop in the serum of guinea pigs immunized with either normal or irradiated cercariae. The lethal antibody response paralleled the immune status of the animal only in the irradiated vaccine model. Guinea pigs vaccinated with highly irradiated cercariae of *S. mansoni* and analyzed for their ability to kill challenge parasites showed consistently that lung schistosomula and 2-week-old parasites were killed preferentially by sensitized animals, but that older worms were refractory.²³⁷ This implied that liver phase immune elimination is a stage-dependent phenomenon in the vaccine guinea pig model of schistosomiasis.

More studies have addressed the humoral and cellular basis of specific immunity in the guinea pig irradiated vaccine model of schistosomiasis.²³⁸ Serum obtained from once-vaccinated animals conferred modest levels of resistance to naïve recipients, but this was not consistent. Serum from vaccinated and thrice boosted rodents routinely transferred around 45% immunity in naïve animals. Neither peripheral lymph node cells nor splenocytes from the polyvaccinated serum donors were able to transfer resistance to recipient guinea pigs, and they failed to augment the protection achieved by polyvaccinated serum. Polyvaccinated guinea pig serum failed to protect mice. Similarly, guinea pigs could not be protected with polyvaccine rat serum that conferred 75% resistance to naïve rats.

Rabbits

Rabbits have been used in feasibility studies that tested saliva for *S. japonicum* diagnosis, which would be a useful noninvasive procedure.²³⁹ Liver pathology due to *S. japonicum* and *S. mansoni* has also been studied in rabbits with regard to worm burdens and duration of infection. It was shown that pathology of schistosomiasis in rabbits has peculiar aspects compared to human pathology.²⁴⁰ Advanced liver fibrosis in *S. japonicum*-infected rabbits is slowly reversible after cure or senescence of the infection.²⁴¹ Symmers clay-pipe fibrosis of the liver was produced by *S. japonicum* infection in rabbits.²⁴² Gross and microscopic fibrosis and portal vascular lesions resembled those in hepatosplenic schistosomiasis in humans. These studies demonstrated that although the rabbit *S. japonicum* model is an imperfect model of Symmers fibrosis, it is the only model presently available other than the chimpanzee.²⁴² New Zealand white rabbits have also been used to investigate whether schistosomiasis may have an association with renal failure.²⁴³ The findings showed that *S. japonicum* infections in the rabbit offer an excellent model system for studying not only the renal pathology associated with human schistosomiasis but also the pathogenesis of amyloidosis, which is a frequent sequela observed in a variety of chronic inflammatory infections.²⁴³ An *S. mansoni* model of ocular inflammation has also been developed in New Zealand rabbits.²⁴⁴ This model was found useful for analyzing immunological parameters involved in ocular granulomatous and parasitic diseases, humoral and cellular responses mediating auto sensitization to retinal or other ocular antigens, and chemotherapy of host inflammatory responses.²⁴⁴

The rabbit *S. mansoni* model has been used to study concomitant immunity,²⁴⁵ demonstrating that immunization of New Zealand rabbits with an adult worm antigenic extract induced a protective response after challenge infections, as compared to worm reduction due to a second infection, confirming earlier studies.²⁴⁶ Studies had also shown that rabbits exposed to reinfection are able to kill worms from their primary infection, besides having protection against challenge parasites.²⁴⁷ The fatty acid binding protein Sm14 has also been tested for its protective potential in rabbits.²⁴⁵ The ability of fractionated serum from rabbits vaccinated with irradiated *S. mansoni* to transfer immunity to mice has also been studied.²⁴⁸

Dogs

A natural infection with *S. haematobium* was reported in a dog in Zambia.²⁴⁹ Confirmed cases of canine schistosomiasis mekongi in Cambodia have suggested that dogs are animal reservoirs of *S. mekongi* in the survey areas.^{250,251} The role of several hosts in the transmission of *S. japonicum* in one region of China and in the Philippines²⁵² revealed that dogs had the highest rates of prevalence and were the main transmission source and therefore important in the epidemiology of schistosomiasis. Dogs have also been found infected with *S. incognitum*, a blood fluke of a variety of mammals with zoonotic potential.²⁵³ Acquired immunity in dogs infected with *S. incognitum* has been described.²⁵⁴ *Heterobilharzia americana* infection (American schistosomiasis) has also been reported in dogs.^{255–257} Canine schistosomiasis as a result of *H. americana* infection can be diagnosed by indirect haemagglutination tests²⁵⁸ or a miracidia hatching test.²⁵⁹

Dogs have been used in chemotherapy studies designed to evaluate histopathological changes of the liver of animals treated with artesunate or praziquantel during early-stage infections with *S. mansoni*.²⁶⁰ The prophylactic effects of artesunate on *S. japonica* infections have also been studied in dogs.²⁶¹ An early treatment with artemether given in appropriate regimens was tested in rabbits and dogs and shown to be effective for controlling acute schistosomiasis.²⁶² The pathological changes in liver and intestines of rabbits, dogs, and cats after *S. japonicum* infection have been studied and indicated that pathological lesions varied in different animal species.²⁶³

Pigs

Recent data on the pig indicate that this natural host of *S. japonicum* might be a realistic alternative to rodent and primate models, as recently reviewed by Johansen and coworkers.²⁶⁴ As in humans, *S. japonicum* establishes mainly in the large intestinal veins, with high fecal egg counts during the acute phase of infection, which vary greatly within and between days. Concomitant immunity is another shared feature, but studies in pigs have indicated that the phenomenon is more complex than generally thought. Clinical signs such as eosinophilia and diarrhea with mucus and blood in the acute phase of infection and hepatomegaly, increased portal diameter, periportal fibrosis, and ascites in chronic infections are common findings in both humans and pigs. A low-protein diet aggravated the disease in pigs by increasing the establishment rates, fecal egg excretion, and morbidity. A 100% cure rate is achieved when *S. japonicum*-infected pigs are treated with praziquantel at 40 mg/kg, and 4 weeks post treatment pigs remain resistant to reinfection. Human congenital *S. japonicum* infections have been confirmed in pigs, but the implications of such infections for the pathogenesis of *S. japonicum* remain to be investigated.

Nonhuman Primates

Several animal models have been used to study the basic biology, immunology, and pathogenesis of schistosomiasis. Rodents and particularly inbred strains of mice tend to be the animals of choice in experimental *S. mansoni* infection, essentially because of their easy availability, fast breeding, and development of disease on experimental infection. In this regard, they have contributed greatly to our understanding of human *S. mansoni* infection. However extrapolation of data from mice to humans is not without problems, as obvious anatomical, genetic, and immunological differences exist between the two species. In addition, murine studies have been conducted in highly inbred strains of mice and might not reflect the heterogeneity of response observed in human infections. Primates in many ways are better than rodents, although they are restricted by ethical standards and are expensive for general use. Primates, however, remain invaluable for testing the relevant rodent findings to man.

An excellent review on the use of primates in schistosomiasis was written by Sturrock.²⁶⁵ Historically, studies of African schistosomes were reported in monkeys in Egypt. Green monkeys

of West African origin were found naturally infected with *S. mansoni* in the West Indies. Natural infections have since been reported in other primates, including baboons and cercopithecine monkeys. They appear to be anthroponoses, with the monkeys becoming accidentally infected in areas of endemic human transmission, but humans did not seem to be involved in one focus in Tanzania and possibly another in Kenya. Many primate species have been successfully infected experimentally with *S. mansoni*, which is the parasite most amenable to laboratory maintenance. Rhesus monkeys (*Macaca mulatta*) capuchine monkeys (*Cebus apella*), baboons (*Papio* spp.), and various cercopithecine monkeys (*Cercopithecus aethiops*) have all proved susceptible. Other less commonly available primates ranging from tree shrews to chimpanzees have also been experimentally infected with *S. mansoni*. Vervet, rhesus, capuchin, and taloipin monkeys, baboons, and chimpanzees are also susceptible to *S. haematobium* and *S. japonicum* (see Sturrock²⁶⁵ for references). Some salient features of three most commonly used primates in schistosomiasis research are given below.

Rhesus Monkeys

Studies of schistosome infections using rhesus monkeys have been reported from as far back as 1953.²⁶⁶ Rhesus monkeys have been shown to be susceptible to other nonhuman schistosome parasites including *S. incognitum* and *Orientobilharzia dattai*.²⁶⁷ Parasitological and histopathological studies have been carried out in rhesus monkeys infected with *S. japonicum*.²⁶⁸ Cellular and humoral responses of rhesus monkeys have been studied after primary and chronic *S. mansoni* infection, and protective immunity was correlated to activation of both cell-mediated and humoral mechanisms.^{269,270} A study was undertaken in rhesus monkeys to examine the potential role of immunodiagnostic methods in determining successful chemotherapy during schistosomiasis.²⁷¹ This study allowed for isotypic analysis of antibody responses and demonstrated that all isotypes increased after praziquantel treatment. Immunization experiments have been performed in rhesus monkeys to demonstrate the efficacy of a Kenyan strain of *S. mansoni* against challenge infections in rhesus monkeys, which compared favorably with observations in baboons.²⁷² The resistance of rhesus monkeys after immunization with irradiated cercariae was shown to be IgE-, mast cell-, and eosinophil-dependent.²⁷³

Baboons

Baboons are the most extensively used nonhuman primates and are the models of choice for schistosomiasis research. They are anatomically, genetically, and immunologically more similar to humans than are rodents. At the genetic and protein levels, a 92–99% homology exists between baboons and humans.²⁷⁴ Baboons are natural hosts for *S. mansoni* in East Africa and are highly susceptible to experimental infections.^{275,276} Infection of baboons with *S. mansoni* represents a model of disease and acquired immunity with closer parallels to humans than mice (reviewed by Nyindo and Farah²⁷⁵). For example, in baboons as in humans but unlike mice, parasite-specific IgG and IgE are associated with protection.^{204,276} In addition, baboons infected in the wild show an age-dependent prevalence, with high rates of infection in juveniles and young adults. Due to their greater body mass, an experimental infection of sufficient size to evaluate vaccine efficacy does not represent such a massive antigen exposure. Even a single worm pair in a mouse represents a much heavier infection than would ever be encountered in humans. Baboons adapt readily to changes in their environment, can give birth twice every 18 months, can live for over 20 years, and can attain body weights of >20 kg. It is also possible to monitor disease in multiple organs throughout the course of infection, by surgical manipulation.^{277,278} Adjuvants can be safely applied in baboons, unlike in rodents where they may generate a different type of immune response. The use of appropriate adjuvants and vaccine delivery systems is now recognized as crucial in the design of successful subunit human vaccines since the immune response can be manipulated to generate

the appropriate type of effector mechanism. Finally, many of the necessary reagents to evaluate the fine specificity of immune responses to vaccines are now available for baboons.^{278,279}

Five types of baboons have been described (olive, yellow, red, chacma, and sacred), and they have been divided into two species: *Papio hamadryas* and *P. cynocephalus*. *Papio cynocephalus* comprises *P. cynocephalus cynocephalus* (yellow baboon) and *P. c. anubis*. A summary of the salient features of baboon infection with *S. mansoni*, such as its permissiveness to infection, parasitological parameters, clinical disease and pathology, and immune responses, is presented below and was recently reviewed by Nyindo and Farah.²⁷⁵

Baboons are natural hosts for *S. mansoni* and can both maintain and transmit infection in the wild. One study in the endemic area of Kibwezi, Kenya reported over 40% of stool from 85 baboons as positive for *S. mansoni* eggs and 46% of 146 perfused baboons positive for adult worms. In baboon experimental *S. mansoni* infection, there is a high rate of cercarial penetration, fast schistosomula migration from the skin to the lungs and from the lungs to the liver, and development to adult worms. Maturation of infecting cercariae often exceeds 90%, compared to 50% in mice. The prevalence of infection in baboons was studied at the Gombe National Park, Tanzania, where animals were believed to harbor recently acquired *S. mansoni* infections. The study showed that prevalence of infection rises from a very young age, peaks at age 2 to 3 years (juveniles and young adults) and then declines, in a similar manner to that reported in humans.

In baboon infections of *S. mansoni*, adult worms pair up and start to lay eggs about 5 to 6 weeks post infection. Fecal egg counts are high during the acute phase of the disease and decline as the chronic phase sets in. Fecundity, the number of eggs excreted per worm pair per day (epg), is higher in baboons than in murine infections. In one study²⁷² the fecundity of a Kenya strain of *S. mansoni* was 1107 epg, compared to 234 to 380 epg in the mouse. In infected humans, tissue eggs are most abundant in the large intestine,²⁸⁰ and the tissue egg distribution in baboons is more similar to that seen in humans than in mice. In baboons, about 80% of eggs lodge in the intestines and only 10 to 20% are present in the liver, whereas in mice, approximately 77% of eggs are trapped in the liver and only 20% are in the intestines.

As in humans and mice, two discrete phases of disease progression have been described in baboons; the acute phase commences from around 6 weeks and can stretch to up to 12 weeks post infection depending on cercarial dose, mode of infection, and immune status of the animal. During the acute phase, infected baboons become anorexic, lose weight, and develop diarrhea and occasionally, bloodstained feces are seen. Large liver and intestinal granulomas are associated with this phase of disease. The second phase is the chronic stage, characterized by resolution of granulomatous pathology and amelioration of hepatointestinal disease. Hepatic fibrosis associated with chronic schistosomiasis has been demonstrated in chronically infected baboons. There is a more rapid modulation of schistosome egg granulomas in the baboon compared with the mouse. During the early acute phase of disease, *S. mansoni* egg granulomas are composed predominantly of eosinophils, lymphocytes, and macrophages. Once modulation sets in, the predominant cell types are mononuclear cells. Unlike the murine model, where infection invariably leads to development of a florid granulomatous inflammation and fibrosis, fibrosis is not always present in infected baboons. Thus, the baboon exhibits pathological manifestations that more closely parallel the disease in humans, where clinically significant fibrosis only develops in fewer than 10% of heavily infected people. Fibrosis fails to develop in baboons that receive single or repeated infections with *S. mansoni* parasites, although it was observed in long-term chronic infections and in animals exposed to multiple infections and treatment with praziquantel. Anatomically, fibrosis that is observed in baboons and humans differs from that seen in mice. While it is primarily periportal in mice, in the baboon most fibrosis occurs within the portal tracts. Most fibrosis in humans also develops primarily within portal tracts to form the characteristic periportal or pipe stem fibrosis evident in a proportion of infected people.

The immune responses that accompany infection of baboons with *S. mansoni* have been studied extensively. The immune response generated in baboons during acute disease is characterized by

a mixed Th1/Th2 cytokine response, which contrasts with the Th1–Th2 switch that is observed during peak granuloma formation in murine models. The granuloma modulation correlated with an increase in serum soluble egg antigen-specific IgG and IgE (but not IgM) levels. It appears that granuloma development and modulation in baboons might involve antibody-mediated mechanisms, along with sequential induction and diminution of mixed cytokine responses.

Similar to murine and human infections, baboons also develop resistance to infection with *S. mansoni*, particularly after repeated small doses of cercarial challenge. Unlike with mice, this protection is not a consequence of the challenge infection bypassing the liver via porto-systemic collateralization.^{276,281} Instead, this protection is thought to be immunologically mediated and related to increased production in the serum of adult worm-specific IgG. Resistance to reinfection has also been demonstrated in baboons after treatment with praziquantel, which is similar to human studies of reinfection after treatment.²⁸² This resistance correlated with levels of soluble worm antigen preparation-specific IgE antibodies.

Despite the many outstanding qualities of baboons as a model of choice for schistosomiasis studies, there are a number of drawbacks:

1. Baboons are expensive to acquire and maintain in captivity.
2. The services of highly qualified personnel are required to look after their health and perform surgical manipulations.
3. A need still exists for suitable immunological reagents that would widen the scope of studies.

Chimpanzees

Higher primates such as chimpanzees are suitable models for studying schistosomiasis but as endangered species should only be used for critical studies and when lower nonhuman primates are unsuitable.²⁶⁵ Natural infection of chimpanzees with *S. mansoni* was reported in Sierra Leone and diagnosis confirmed by the presence of eggs and larvae.²⁸³ Experimental infections of chimpanzees with *S. japonicum* were reported by von Lichtenberg in 1971,²⁸⁴ while those of *S. haematobium* in chimpanzees have also been reported.^{285–287} Sadun²⁸⁷ also reported the parasitological, radiological, biochemical, and pathological aspects associated with evolution of schistosomiasis in chimpanzees. Parasitological sampling methods have been worked out for experimental animals including chimpanzees.²⁸⁸ Chimpanzees experimentally infected with *S. japonicum* develop circulating schistosome antigens after exposure, and the persistence of these antigens was postulated to result in the observed renal damage.²⁸⁹ In other studies, it was shown that chimpanzees and gibbons could be experimentally infected with *S. intercalatum* to produce disease and pathology, although no histopathology was detected in the urogenital system.²⁹⁰ Experimental renal disease due to schistosomiasis has been reported in primates, including chimpanzees.²²⁴

With regard to pathology, the hepatic, intestinal, and cardiopulmonary lesions produced by *S. mansoni*, *S. haematobium*, and *S. japonicum* in humans and experimental animals including chimpanzees were shown to bear striking similarities but usually have distinctive features as well.^{280,290} Thus while egg deposition and immunoregulation produce Symmers fibrosis in chimpanzees after *S. mansoni* and *S. japonicum* infection, no such fibrosis is observed in *S. haematobium*-infected chimpanzees despite the presence of equivalent liver egg burdens.^{280,290} The effects of portacaval shunting on *S. japonicum* infection were studied in chimpanzees, showing that schistosomal nephropathy in chimpanzees is more closely related to infection intensity per se.²⁹¹ The use of chimpanzees in the study of nephropathy of hepatosplenic schistosomiasis has also been reported.²⁹² The association of chronic hepatitis and schistosomiasis has been investigated in chimpanzees, the findings indicated that schistosomiasis has the potential to induce chronic hepatitis and that the latter condition is not always due to chronic viral infections.²⁹³ This followed the first reports of hepatocellular carcinoma associated with *S. mansoni* in a chimpanzee.²⁹⁴

Immunological reactions have been studied in chimpanzees experimentally infected with *S. japonicum*.²⁹⁵ Early vaccination studies looked at administration of X-irradiated cercariae of *S. japonicum* and the resultant pathology.^{296,297} The radiation-attenuated vaccine, which is highly effective in rodents and primates, was recently tested in the chimpanzee in order to strengthen its status as a paradigm for human recombinant antigen vaccination.²⁹⁸ These studies revealed that the antibody responses in vaccinated chimpanzees were dominated by responses directed at glycosylated epitopes on parasite antigens.^{298,299} Certain plants fed on by chimpanzees have been shown to contain compounds that have antischistosomal activity; these are possibly used by chimpanzees to control against parasitic infections.³⁰⁰

HUMAN AFRICAN TRYPANOSOMIASIS (HAT)

Etiology and Transmission

Trypanosomiasis is a zoonotic vectorborne disease caused by protozoan parasites of the genus *Trypanosoma*. Of the different species, *T. brucei rhodesiense* and *T. b. gambiense* infect and are pathogenic to man. The resultant human African trypanosomiasis (HAT) or sleeping sickness leads to a protracted, debilitating, and finally fatal disease in untreated cases. *Trypanosoma brucei rhodesiense* produces an acute disease and is mainly found in eastern and southern Africa, while *T. b. gambiense* produces the chronic form of the disease, with an incubation period sometimes as long as 2 to 10 years, and is to be found in west and central Africa; the disease is commonly called Gambian trypanosomiasis. HAT is found in sub-Saharan Africa within the vector tsetse fly (*Glossina* spp.) belt. The disease was brought to controllable levels in the 1960s, but a resurgence of sleeping sickness has occurred in large portions of Africa, with 350,000 to 450,000 persons now estimated to be infected (Figure 9.3).^{301–303} In some of the regions, the incidence of trypanosomiasis has increased faster than that of human immunodeficiency virus (HIV)/AIDS, with epidemic levels currently present in Democratic Republic of Congo, Angola, Sudan, and Uganda. Over 60 million people are considered at risk of infection in the 36 countries in which HAT is endemic. The disease runs a complex course initiated by the bite of an infected tsetse fly. The parasites multiply at the chancre and invade the hemolymphatic system and later spread to different organs. This is classified as the early stage. Some of the organs that are particularly susceptible are the heart and central nervous system (CNS). The involvement of the CNS, characterized by invasion of the brain and spinal cord by trypanosomes and abnormal cerebrospinal fluid (CSF), is usually the culmination of the untreated early-stage disease and is called late-stage or meningoencephalitic stage, characterized by episodes of sleep at odd times of the day. It is from the latter that the disease derives its common name — sleeping sickness. An early description of the disease reads as follows: “It was in a way very merciful death — though slow — for it was without pain, turning from a fitful form into unconquerable laziness strange to see in the busy natives, it passed, this death, from lethargy into ridiculous sleepiness that made their mouths fall open while they ate; it went at last from such drowsiness into delirious coma — no waking from this — and into a horrible unnatural coldness that emerged with the chill of the grave. Such was the African sleeping sickness.”³⁰⁴ Most patients are presented for treatment when the CNS is already involved,³⁰⁵ complicating the choice of therapy.

Diagnosis of HAT by demonstration of trypanosomes in peripheral circulation or CSF does not always give the true picture of the infection status. The discovery of clinical and biological clues that herald the involvement of the CNS assumes major importance in determining the choice of therapy. The World Health Organization (WHO) recommends a follow up of between 24 and 36 months for treated patients to establish cure.³⁰⁶ This follow up for patients treated during late-stage trypanosomiasis is lengthy, involves painful lumbar punctures, and is expensive due to travel costs. As a result, there is poor compliance by patients, who discontinue follow up as soon as they start

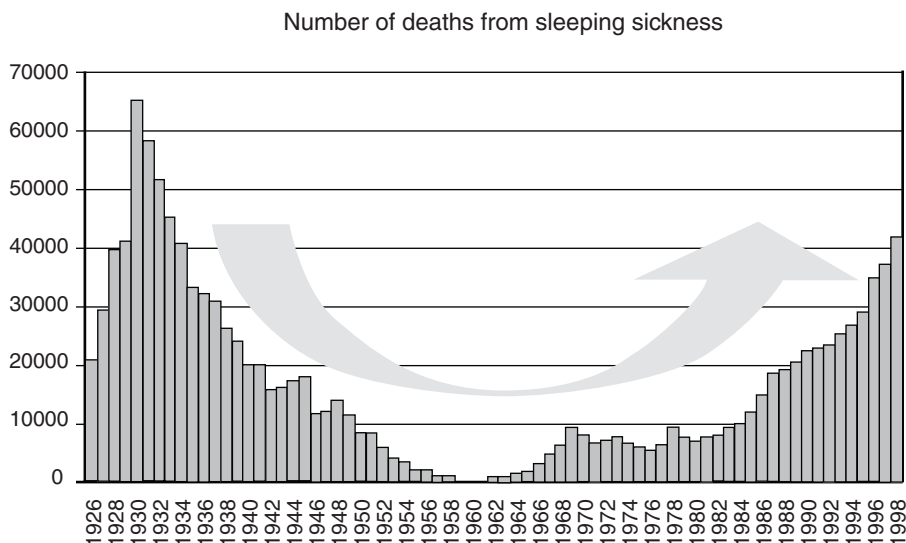


Figure 9.3 Deaths due to sleeping sickness during the 20th century (www.eanett.org).

feeling better. Consequently, the search continues for an alternative diagnostic procedure that is cheap, less invasive, and easy to perform under field conditions and requires a shorter follow-up period.^{306,307}

Successful chemotherapy necessitates the use of drugs that will cross the blood-brain barrier. The early stage drugs, suramin (e.g., Germanin®, Bayer, Germany) and pentamidine (e.g., Penta-carinate®, May and Baker, U.K.), are effective and less toxic and have fewer instances of drug resistance. The treatment of late stage HAT mainly depends on the arsenical drug melarsoprol (Arsobal®, Specia, France). This drug is associated with severe post treatment reactive encephalopathy (PTRE) in about 5 to 10% with a fatal outcome of 1 to 5% in treated patients.^{308,309} There are also recent reports of treatment failure rates with melarsoprol of over 25% in endemic areas of *T. b. gambiense* in northern Uganda and northern Angola.^{310–312} Worse still, continued manufacture of this drug is unlikely due to technical and major ecological concerns associated with its synthesis.³⁰³ D,L- α -Difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase, has been successfully used in the treatment of late-stage *T. b. gambiense* infections.³¹³ However, its use in the field is hampered by lack of effectiveness against *T. b. rhodesiense* infections and the prohibitive cost during administration of the drug to patients.³⁰⁹ Consequently, combination therapy of new and existing drugs continues to be a priority area for research.^{307,314,315} The WHO, in collaboration with manufacturers of the currently used drugs, has recently agreed on a plan to make the drugs available to the patients up to 2005/6, and all national governments have to do is pay for the freight charges to the point of need. There is therefore an urgent need for further research into newer drugs for treatment of sleeping sickness, especially those targeting the second-stage form of the disease.³⁰⁷ The need for animal models to study the various aspects of the disease is crucial. The following induced laboratory animal models have been used variously in generating useful information on diagnosis, pathogenesis, and treatment of sleeping sickness.

Nonhuman Primates

Since establishment in the Kenya Trypanosomiasis Research Institute (KETRI) in 1982,^{316–319} the vervet monkey (African green monkey, *Cercopithecus aethiops*) has been an invaluable model for investigations on disease pathogenesis^{320–323} and chemotherapeutic studies of potential trypanocidal compounds.^{324,325} The vervet monkey model was fundamentally started with the particular

aim of establishing a disease syndrome in monkeys caused by trypanosomes that is identical to sleeping sickness in humans, particularly the stage where the brain is affected, called meningoencephalitis. Currently, when vervet monkeys are experimentally infected with 10^4 *T. b. rhodesiense* KETRI 2537, the disease produced is classified into three models:³¹⁹

1. The early-stage disease model formed by the infection between days 0 and 35
2. The terminal disease model, in which the infection is allowed to develop to 42 days post infection (dpi)
3. The advanced late-stage model, in which the infection is treated on days 33 to 35 with diminazene aceturate and allowed to relapse 50 to 70 days later (80 to 100 dpi)

Trypanosomes are detected in the cerebrospinal fluid (CSF) by day 7³²³ after infection and increase in number with progress of the disease.³²⁰ The vervet monkey model therefore provides an excellent opportunity for studies on the disease not only in the early hemolymphatic stage but also, and more importantly, the stage when trypanosomes have entered the CNS, resulting in the late, meningoencephalitic stage.

In this model, drugs that can cure the early stage of the disease include diminazene aceturate and suramin. Diminazene aceturate is curative up to 28 dpi, when administered at 5 mg/kg intramuscularly for three consecutive daily doses. Suramin is also curative when a regimen of 20 mg/kg is instituted over a 10-day period such that the animals receive the drug at days 1, 3, 5, 7, and 10 of treatment.³²⁶ Melarsoprol is the only curative drug for late-stage disease in this model, when administered daily at 3.6 mg/kg intravenously on four consecutive days. The current recommendation when using this model is to monitor the experimental animals for at least 600 days post infection to ascertain cure.

The main drawback of the model is that the animals are wild caught and although monitored for diseases, including zoonoses, during the quarantine period, it is not possible to ascertain the specific disease status of the animals or possible exposure while in the wild.³²⁷ However, the data obtained have been shown to be consistent, with no variation over time in individual animals even where variations between animals may be marked.³²⁸ Second, the use of diminazene aceturate to induce the late-stage disease introduces factors that may complicate the analysis of the data, especially if a diamidine is being tested using the model. There is therefore a need for a continued search for a chronic meningoencephalitis model that does not require pretreatment with early-stage drugs.³²⁹ The use of serological tests has been investigated in the past to determine possible cut-off points in order to shorten the lengthy post-treatment follow-up period.³³⁰

Mice

Much of what is known about HAT has been achieved through the vast number of studies carried out in the mouse model, which has become an invaluable tool in pathogenesis and drug testing. Most *T. brucei* trypanosomes usually produce an acute disease in mice, a factor that has been a major setback when using these animals for the study of the more chronic form of the disease as it occurs in humans.³³¹ A variety of chronic mouse models of the disease have been developed by a number of workers in different laboratories. Broadly, the models can be divided into those using *T. b. rhodesiense* or *T. b. brucei*. It is important to point out that it is rare to obtain a model that completely resembles the human form of the disease.

Mouse Models Using *T. b. rhodesiense*

Only a few workers have recorded development to chronic form of the disease in mice using the human infective parasite. A stablate of *T. b. rhodesiense*, which was isolated from a patient in the early stage of infection at the East African Trypanosomiasis Research Organisation (EATRO),

Tororo, Uganda, in 1972, was first studied in 1979.³³¹ This stabilate, EATRO 1989, was inoculated into a vervet monkey, which developed a chronic infection of more than 4 months. This strain was further investigated in outbred female mice, which were observed to develop a chronic infection lasting 6 to 9 weeks. A meningoencephalitis, beginning with meningitis 1 week after infection and being fully developed after 4 weeks, was demonstrated.³³¹ It should be noted here that other *T. b. rhodesiense* stabilates ordinarily produce an infection that lasts 1 to 2 weeks. The inoculum dose used was 1×10^5 obtained from a donor mouse immunosuppressed by gamma-irradiation. KETRI 2537, which was derived from EATRO 1989, was later used to develop chronic models in vervet monkeys and Swiss white mice at KETRI, Muguga, Kenya.

In all the studies, the CNS inflammatory reactions observed corresponded to a certain degree to those known to occur in the late stage of human sleeping sickness in their nature and way of spreading.³³¹ They are primarily meningitis followed by encephalitis, which is produced through spreading of the inflammatory reactions from the meninges via the invading blood vessels. The inflammatory infiltrations are composed mainly of lymphocytes and plasma cells. However, despite the occurrence of these neuropathological changes in this model, little is documented on the occurrence of clinical signs related to the disease pattern. When using the model to screen for late-stage drugs, the mice are kept untreated for 21 days after infection. A 180-day post-treatment follow up on parasitemia is recommended before cure can be declared. Mice dying before the post-treatment period are subjected to histopathological examination.

This model has a major pitfall in that only meningitis has been observed at 21 days post infection, with meningoencephalitis setting in after 28 dpi, when a substantial number of mice may have died. It is thought that animals with meningitis alone are in a transitional stage and can sometimes be treated successfully with early-stage drugs. The mouse has a different physiological environment when compared to the human, and as such some of the findings cannot be directly extrapolated.

Mouse Models Using T. b. brucei

The parasite *T. b. brucei* closely resembles the human-infective trypanosomes but is not pathogenic to humans. This has made it a more popular and acceptable choice for studies in laboratories found outside the disease-endemic countries, due to the reduced risk to the workers. The *T. brucei* stock used was originally isolated from a wildebeest in the Serengeti park, Tanzania, in 1966. A clone from this stabilate GVR 35/C1 has been used in various studies. Mice are infected intraperitoneally with 2×10^4 to 4×10^4 expanded parasites in sublethally gamma-irradiated mice. Infected mice develop characteristics that resemble the human infective forms.^{332,333} As with *T. b. rhodesiense*, meningoencephalitis is rare with *T. b. brucei* unless it is induced by use of early-stage drugs.^{334,335}

When inoculated with *T. brucei*, commonly through the intraperitoneal route, the mouse develops a parasitemia within 3 to 4 days. The meningoencephalitis stage of the disease, which is the major point of interest, is induced by treating the mice with drugs that do not cross the blood-brain barrier (BBB), such as diminazene aceturate³³⁴ or suramin.³³⁵ Successful induction of meningoencephalitis by *T. brucei* suggests that trypanosomes localize early in the disease process in areas with reduced BBB, such as the choroids plexus and perivascular spaces, favoring further migration of the trypanosomes into the CSF and further invasion of the brain tissue.^{334–336}

The mouse model, as a small vertebrate, provides the first *in vivo* testing stage after screening *in vitro* of potential compounds. Using this model, a variety of novel drug candidates and drug combinations have been tested.^{315,337–342} The mouse is amenable to genetic manipulation to produce knockout mice of desired genetic makeup to suit particular studies. Using this method, major pathways of the disease process have been studied.^{343–348} With the few drugs available for treatment of sleeping sickness and the need for better diagnostic tools, the mouse stands out as an important laboratory animal for continued research.

Rats

Different strains of rats have been used in various trypanosome studies. However, experimental studies on sleep disorders have been fundamental. Experimental infection with the parasite *T. b. brucei* in the rat (*Rattus norvegicus*) provides a unique and robust model of dysfunction of sleep regulatory mechanisms in a structurally intact brain because the length of synchronized sleep episodes is selectively and dramatically reduced in the advanced stages of the disease.^{349–353} The studies, carried out in the Sprague–Dawley or Wistar stocks, have shown that the suprachiasmatic nucleus, which plays a critical role in the biological clock entraining endogenous rhythms in the mammalian brain, is impaired during infection with trypanosomes, resulting in disruption of the endogenous melatonin secretion and binding activity.^{354,355} This may be accompanied by cytokines and chemokines that either enhance parasite growth or suppress the immune response, at least in the early stages of the disease.^{356–359} The role of endotoxins in the induction of neuropathology has also been studied in the rat, and it is hypothesised that the breakdown of the blood-brain barrier is partly due to a “leaky” gut in trypanosomiasis that leads to increased levels of circulating endotoxins, mainly from the Gram-negative bacteria in the gut.^{361,362} While most studies have been directed towards understanding the sleep dysfunction from which the disease derives its name, the role of the rat model in other disease aspects such as reproduction,^{363–365} disease process,^{366,368,369} isolation, and diagnosis,³⁶⁷ as well as drug research^{370,371} and behavior,³⁵⁹ is also important.

Dogs

Dogs have been used to study the human disease. The breed of choice is beagle, but dogs of indeterminate breeds have also yielded good results. When dogs are experimentally infected with *T. brucei*, they show an acute disease pattern that develops rapidly, resulting in death within the first wave of parasitemia around 7 and 8 days post infection (dpi). Some dogs, however, develop a subacute infection that results in terminal disease between 24 and 28 dpi, corresponding to the second wave of parasitemia.^{372,373} The general picture is that of loss of condition, ocular impairment, elevated temperature and pulse and respiratory rates; the difference between the acute and subacute diseases lies in the degree of intensity. The infected animal also develops anemia, monocytosis, panleukopenia, and thrombocytopenia, suggesting that a bone marrow–depressing factor exists in the plasma of *T. brucei*-infected dogs.^{374,375} A number of studies have concentrated on the heart, where it has been observed that a marked reduction in the plasma concentration of atrial natriuretic factor (ANF), a polypeptide hormone secreted by the heart muscle cells, occurred in the terminal stage of the disease during weeks 3 and 4 post infection.³⁷⁶ By impairing the ability of the heart and kidneys to regulate blood volume, the alterations in ANF and plasma rennin activity (PRA) could be involved in the pathogenesis of heart failure in *T. brucei*-infected dogs. Knowledge of the properties of the hormone would provide insights into the pathophysiology of important clinical entities, such as the pancarditis seen in HAT,³⁷⁷ and could lead to the development of new pharmaceutical products. The dog as a model of HAT provides an opportunity for such studies.

Chickens

Although chickens have been regarded as not susceptible to trypanosomiasis, experimental infection with avian and mammalian infective trypanosomes produced pathology of varying degree. Domestic chickens (*Gallus domesticus*) have been shown to be susceptible to *T. b. brucei* and *T. b. rhodesiense*. When domestic chickens were experimentally infected with *T. brucei* Lugala/55/EATRO/459, they regularly developed a chronic infection,³⁷⁸ which was characterized by a low intermittent parasitemia (3 to 100 parasites per ml of blood) that lasted for 9 months in pullets and over 1 year in cockerels. The level of parasitemia was dependent on the age of the bird at infection.³⁷⁹ Histological examination of spleens at different stages of the infection showed that

the infection produced a progressive increase in the number of germinal centers during the early stages of the infection, with peak levels attained at Day 84.³⁸⁰ These observations indicate that the development of such a large number of germinal centers reflects the bird's response to the elaboration of a succession of trypanosome variable antigens. This in turn would suggest that it is possible to design effective immunology studies in birds for application in mammals, where pathogenic trypanosomiasis occurs. Avian trypanosomes are also pathogenic, and studies carried out demonstrated pathological changes similar to those observed in mammals infected with salivarian trypanosomes.³⁸¹ The ability of three species of avian trypanosome, *T. corvi*, *T. bouffardi*, and *T. everetti*, to protect against heterologous and homologous challenge has been studied in susceptible birds. None protects against challenge with either or both of the other two species, and mixed infections can be obtained experimentally.³⁸² Similarly, domestic chickens infected with a stock of *T. brucei* exhibited a chronic infection that terminated in self-cure and were thereafter immune to challenge with derivatives of the same trypanosome stock.³⁸³

The chicken is increasingly becoming an important refinement tool in laboratory animal science due to the ability to produce antibodies to various pathogens without invasive procedures.³⁸⁴ This relevance has not been realized in the field of trypanosomiasis research.

Animal Models for *T. b. gambiense*

Trypanosoma brucei gambiense generally causes a chronic disease in humans. The subspecies *T. b. gambiense* is divided further into groups 1 and 2. Group 1 *T. b. gambiense* conform to the classical concept of *T. b. gambiense*, which runs a chronic course in humans. It is widespread in tropical Africa eastwards from Senegal to Zaire, southwestern Sudan, and northwestern Uganda. Group 2 *T. b. gambiense* is more virulent and forms a focus of Gambian sleeping sickness in Gambia. It could also be seen as a West African form of *T. b. rhodesiense*.

The parasite has low virulence in laboratory rodents.³⁸⁵ The following laboratory animals, listed in decreasing order of susceptibility, have been tested: hamster, guinea pig, rabbit (also intratesticular), rat, and mouse. In immunocompetent rodents, *T. b. gambiense* infection leads to low parasitemia that is sporadic but persistent.³⁸⁶ For immunopathological studies, this would be acceptable and various rodents including mice have been used in such studies.^{369,387,388} *Mastomys natalensis* was found to be a better model for *T. b. gambiense* than rats and mice³⁸⁹ and has been extensively used.^{390,391} *Microtus montanus* is hard to breed and difficult to handle. Parasitemia is low, the animals bleed very poorly, and small volumes of blood can be expected. *Mastomys natalensis* is easy to breed and handle, develops a higher parasitaemia than *Microtus*, and is easy to bleed. *Grammomys surdaster* is harder to breed and maintain than is *Mastomys*. The handling is difficult (the animals jump) and decent but fluctuating parasitemias can be obtained. As an alternative to these rodent species, SCID mice have been used. SCID mice do not have functional T- and B-lymphocytes³⁹² and were reported to be highly susceptible to infection with *T. b. gambiense* isolates,³⁸⁶ which provides a useful model for *in vivo* drug sensitivity studies. The use of SCID mice is restricted, however, by the cost of breeding and special housing in isolators.

The low virulence impedes analysis of biological properties of the parasite because of the difficulty of preparing a sufficient amount of parasites. Susceptibility of rodents to trypanosomes has been improved by immunosuppression using chemical compounds or irradiation.^{369,393} With immunosuppression by cyclophosphamide (200 mg/kg i.p.) or dexamethasone (5 mg/l in drinking water *ad libitum*), decent parasitemias of about 10⁷/ml could be obtained. Radiation facilities are not always available, and certain variant antigenic types of trypanosomes do not grow well in irradiated rodents. In other studies, promising results have been obtained in a range of immunosuppressed mice including C57black, Swiss white, BALB/c, and C3H mice.³⁹⁴

Attempts to develop a nonhuman primate model of the disease have only yielded limited success. Infection of vervet monkeys with *T. b. gambiense* isolates resulted in parasites in the CSF 107 days post infection but without any noticeable clinical deterioration apart from enlargement of the spleen

and peripheral lymph nodes noted early in the disease.³⁹⁵ A report of successful experimental infections in the vervet monkey has also been made by Enanga,³⁹⁶ but widespread use of the model has not been observed. Considering that sleeping sickness due to *T. b. gambiense* poses the greater risk to a larger human population, relative to *T. b. rhodesiense*, due to its wider distribution in tsetse- and trypanosomiasis-endemic areas of Africa, the need for more studies to characterize animal models for experimentation and drug studies cannot be overemphasized.

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CHAPTER 10

Animal Models for Muscular Disorders

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INTRODUCTION

Skeletal muscle accounts for 35 to 40% of body weight, is essential for locomotion and breathing, and is a major modulator of cardiovascular function. Individual muscles are made up of muscle fibers, each of which is a multinucleate cell that can be as long as 10 cm in some human muscles. Abnormalities of skeletal muscle that are directly due to disorders of the muscle are termed myopathies, in contrast to those that are due to disorders of the nerve innervating the muscle (the motor neuron), which are termed neuropathies. Disorders of the motor neuron can produce secondary changes in the muscle; for example acute loss of the motor neuron leads to atrophy of the muscle. Thus, diseases such as amyotrophic lateral sclerosis (motor neuron disease) show abnormalities in the skeletal muscle. This chapter deals with animal models for myopathies, which can arise from a number of causes including both inherited and acquired disease and are summarized in Table 10.1.

In many cases animal models for the various human myopathies have been discovered as natural mutants and have been shown to exhibit the same biochemical defect due to mutations in the animal homologues of the genes that cause defects in humans. Where naturally occurring animal models

Table 10.1 Human Myopathies and Selected Animal Models

Classification of Disease	Examples	Selected Animal Models
Hereditary myopathy	Dystrophinopathies Limb girdle muscular dystrophy	<i>mdx</i> , CXMD Various knockout mice
Congenital myopathy	Congenital muscular dystrophy Myotonic dystrophy	dy/dy mouse Various transgenic mice
Ion channel myopathy	Myotonia congenita Hyperkalaemic periodic paralysis Malignant hyperthermia	Adr mouse American quarterhorse Pig
Mitochondrial myopathy		Tfam knockout mouse
Metabolic myopathy	Glyconeogenesis type II	Japanese quail, shorthorn cattle, knockout mouse
Inflammatory myopathies		Experimental autoimmune myopathy
Toxic/iatrogenic myopathy	Cerivastatin myopathy	Dosed rat

have not been found, it has been possible to generate models through transgenic engineering, predominantly through the manipulation of embryonic stem (ES) cells to generate mutations in the murine version of the gene affected in the human patient. Natural and transgenic mutant animals play an important role in understanding aspects of the pathogenesis of the various myopathies as well as serving as important models in which to test a wide variety of therapeutic strategies from pharmacological to gene therapy.

MUSCULAR DYSTROPHY

Duchenne muscular dystrophy (DMD) is the most common inherited myopathy of children with an incidence of 1 in 3500 male births;¹ it appears to affect all racial groups at an equal frequency. It is an X-linked recessive lethal disease that involves muscle necrosis, fibrosis, and muscle wasting, leading to the loss of independent ambulation between ages 7 and 12 years and, without ventilatory support, death in the mid-teens to early twenties. Corticosteroids can modulate the course of the disease to some extent (reviewed in Reference 2), and intermittent positive pressure ventilation can substantially prolong life as the respiratory musculature fails.³ There is a pressing need for more effective treatments for this condition to prevent the progressive muscle loss and associated loss of function.

A number of animal models were used for the early research into DMD including the *mdx* mouse, the dy/dy mouse, the golden retriever muscular dystrophy dog (GRMD, also known as the CXMD) and the dystrophic chicken. This work was revolutionized in 1987 with the discovery of the gene defect responsible for this condition and the identification of the protein dystrophin.^{4,5} This in turn led to the confirmation that the *mdx* mouse⁶ and GRMD dog⁷ were both good biochemical models of the condition, as they lacked dystrophin.^{5,8} The dy/dy mouse has subsequently been shown to be a model for merosin-deficient congenital muscular dystrophy,^{9,10} but the cause of the muscular dystrophy in chickens is still unresolved.¹¹

The *mdx* mouse has become one of the most widely used animal models for a myopathic condition, with over 1000 papers published up to the end of 2003. The *mdx* mouse fails to produce dystrophin due to a point mutation in exon 23 that leads to a stop codon and premature termination of translation.¹² Studies using the *mdx* mouse range from those aimed at understanding the role of dystrophin through to the testing of a range of therapeutic strategies. Therapeutic strategies utilizing genetic approaches include gene repair using chimaeroplasts^{13,14} or short fragment homologous

recombination,¹⁵ antisense-directed exon skipping to bypass the point mutation in exon 23,^{16–18} dystrophin gene transfer using viral^{19–24} or nonviral^{25,26} vectors, and myoblast transplantation.^{27–30} Pharmacological approaches include the use of aminoglycosides such as gentamicin to induce read-through of stop mutations,³¹ the use of protease inhibitors such as leupeptin and MG-132,^{32,33} blocking myostatin to encourage muscle hypertrophy,³⁴ antiinflammatory drugs such as pentoxifylline, and the use of metabolic supplements such as creatine.³⁵ Further details of each of these experimental therapies can be found in a number of reviews.^{36–39}

Although the *mdx* mouse is a good biochemical model of DMD, the pathology is relatively mild. Clinical signs are also mild.^{40–43} The untrained eye would find it very difficult to distinguish between an *mdx* and a wild-type C57Bl10 mouse by simple observation in the cage. Adult *mdx* mice are heavier than controls with clear muscle hypertrophy.^{40,44} Although maximal muscle force is not reduced unless corrected for cross-sectional area, the *mdx* muscle is very vulnerable to eccentric (lengthening) contractions and loses force rapidly after several such contractions.^{45,46} Unlike DMD, where muscle damage is evident at birth, the *mdx* mouse does not exhibit clear muscle damage until age 2 to 3 weeks,^{40–42,44} although some investigators have noted earlier evidence of muscle fragility.^{43,47} There is an initial wave of severe myopathy lasting 4 to 6 weeks (Figure 10.1A), after which the majority of muscle fibers have undergone regeneration, and the rate of necrosis slows but does not cease.⁴⁴ After the onset of the disease, the muscle shows evidence of substantial muscle damage and regeneration, with inflammatory cell infiltrate and very variable muscle fiber size (Figure 10.1B) but in general shows much less fibrosis and fatty replacement of muscle than seen in DMD muscle. The *mdx* only shows muscle wasting and fibrosis of limb muscles late in life.^{48,49} The only exception is the diaphragm in the mouse, where there is extensive fibrosis and muscle wasting (Figure 10.1C and Figure 10.1D) even in young adult mice.⁵⁰ Regenerated muscle fibers can be detected by the presence of central nuclei, which persist for very long periods in rodents. This is in contrast to humans, where the myonuclei migrate to a peripheral position as the regenerated fiber adopts the mature pattern of gene expression. Unlike DMD, the *mdx* mouse maintains a high capacity for muscle regeneration for much of its life. Suppression of regeneration by irradiation of the limb results in the development of more severe pathology resembling DMD.^{51,52} Additional mouse models of DMD have been created by chemical mutagenesis — the *mdx2cv*, *mdx3cv*, *mdx4cv*, and *mdx5cv*^{53,54} — and by gene targeting in ES cells.^{55,56} These all show similar pathology to the *mdx* mouse.

The ability to undertake germ-line genetic correction in mice via transgenesis has allowed experimental analysis of the structure–function relationships for dystrophin and the direct testing of potential gene therapies for DMD. Thus, a number of studies have investigated various forms of recombinant dystrophin for their ability to prevent the development of muscular dystrophy in transgenic *mdx* mice Reference 39. Additional studies have demonstrated similar protective properties when overexpressing several different recombinant forms of utrophin, the autosomal homologue of dystrophin. Overexpression of other molecules has also shown some ability to ameliorate the development of pathology in the transgenic *mdx* mouse, again reviewed in Reference 39. However, all of the transgenic experiments involve the expression of the transgene prior to the development of pathology in the mouse. Two studies, one with dystrophin⁵⁷ and one with utrophin,⁵⁸ have attempted to examine the effect of transgene expression after the development of pathology in the *mdx* using tetracycline-regulated expression of the transgene. Neither study was entirely convincing due to technical problems associated with mosaic expression and concerns about the degree of repression and induction of expression. However, the overall conclusions suggest that both dystrophin and utrophin are good candidates for gene therapy in DMD.

The GRMD dog model of DMD is an alternative to the *mdx* for testing therapeutic strategies. Many of the same approaches taken in the *mdx* mouse have been investigated, including gene repair using chimaeroplasts⁵⁹ and dystrophin gene transfer using adenoviral vectors.^{60–63} In contrast to the *mdx* mouse, the GRMD dog model of DMD shows a very similar phenotype to DMD with clinical signs appearing at 6 to 9 weeks of age.^{64,65} By 6 months of age there is marked muscle atrophy

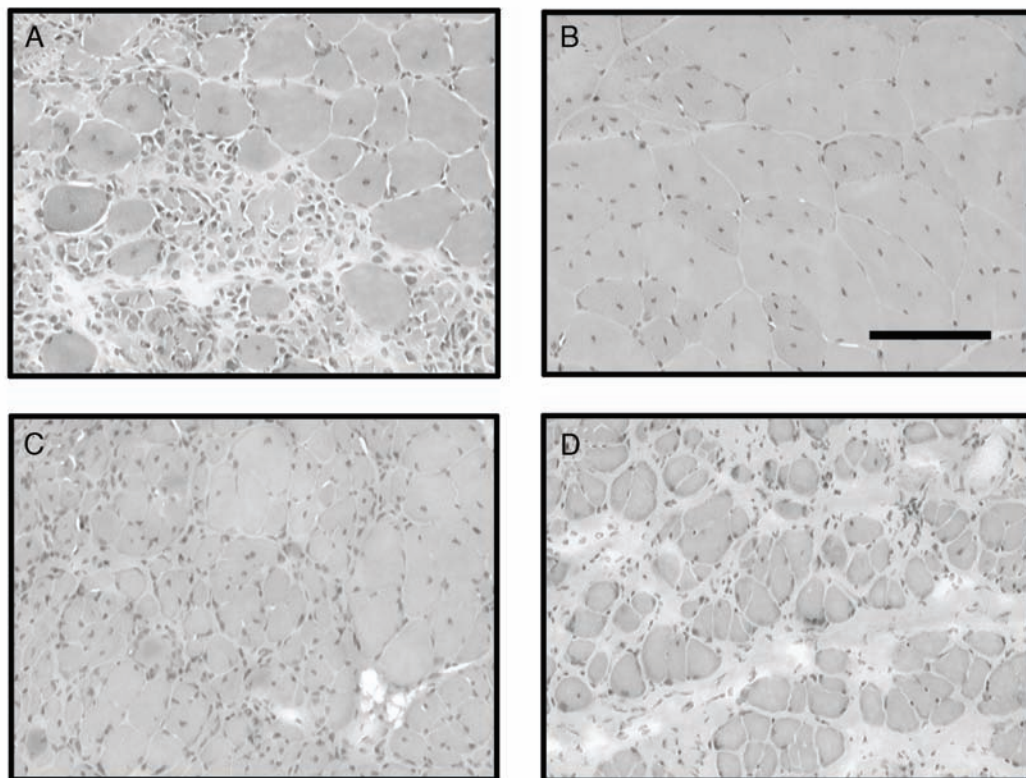


Figure 10.1 Photomicrographs of muscles from *mdx* mice. Panel A shows a transverse section from the quadriceps muscle of a 6-week-old *mdx* mouse. There is focal necrosis with an inflammatory infiltrate. Some muscle fibers have already regenerated and show central nuclei. Panel B shows a transverse section from the quadriceps muscle of a 22-week-old *mdx* mouse. The majority of muscle fibers contain central nuclei, variability in fiber size, and little ongoing necrosis. Panel C shows a transverse section from the diaphragm of the same 22-week-old *mdx* mouse. In contrast to the quadriceps, the diaphragm shows fibrosis, continuing necrosis, and inflammatory cell infiltrate. Panel D shows the diaphragm of a 22-month-old *mdx* mouse; extensive fibrosis and apparent loss of muscle fibers are present. Scale bar represents 100 μ m for all images.

and consequent limb deformity and a head-hanging stance. The muscle shows necrosis, regeneration, and the variable fiber size typical of dystrophic muscle. As the disease progresses, marked fibrosis develops and there is fatty replacement of lost muscle fibers. The loss of dystrophin in the GRMD is due to a splice site mutation leading to skipping of exon 7 and the loss of the open reading frame.⁶⁶ Unlike the *mdx* mouse and DMD cases, many affected pups die shortly after birth with massive necrosis of the respiratory muscles.⁶⁷ A more detailed discussion of the pathology in these animal models of DMD is available in the review by Cooper.⁶⁸

Dystrophin deficiencies have also been described in a number of other breeds of dog including German Shorthaired Pointers.⁶⁹ More recently, an internally deleted dystrophin has been described in the Japanese Spitz breed,⁷⁰ and this animal could be regarded as a model of severe Becker muscular dystrophy (BMD). BMD differs from DMD in that the clinical course of the disease is slower due to the mutation leading to the generation of an internally deleted but still partially functional protein. Cats with dystrophin deficiency have also been described, but the manifestation is substantially different as the tongue and diaphragm become grossly hypertrophied, and the cats have problems with drinking and eating.^{71–73}

After the discovery of the genetic cause of Duchenne muscular dystrophy, the genes associated with many of the other forms of muscular dystrophy have been determined. This in turn has led to the derivation of a large number of animal models, either by identification of defects in existing

Table 10.2 Animal Models of Muscular Dystrophy

Disease	Inheritance	Gene Product	Animal Model	Reference
Duchenne and Becker MD	XR	Dystrophin	<i>mdx</i> mouse, <i>mdx3cv</i> , 4cv, 5cv GRMD dog	12 54 66
LGMD 1B	AD	Lamin A/C	<i>Lmna</i> ^{-/-} mouse	75
LGMD 1C	AD	Caveolin-3	<i>Cav3</i> ^{-/-} mouse	76,77
LGMD 2A	AR	Calpain-3	<i>Capn3</i> ^{-/-} mouse	78
LGMD 2B	AR	Dysferlin	SJL mouse	74
LGMD 2C	AR	γ -Sarcoglycan	<i>Sgcg</i> ^{-/-} mouse	79
LGMD 2D	AR	α -Sarcoglycan	<i>Sgca</i> ^{-/-} mouse	80
LGMD 2E	AR	β -Sarcoglycan	<i>Sgcb</i> ^{-/-} mouse	81
LGMD 2F	AR	δ -Sarcoglycan	<i>Sgcd</i> ^{-/-} mouse BIO14.6 hamster	82 83
Miyoshi myopathy	AR	Dysferlin	SJL mouse	74
Classical CMD	AR	Laminin α 2	<i>dy/dy</i> mouse	9,10
α 7 Integrin CMD	AR	α 7 Integrin	<i>Itga</i> ^{-/-} mouse	84
Bethlem myopathy	AD	Collagen VI α 1	<i>Col6a1</i> ^{-/-} mouse	85
EB and MD	AR	Plectin	<i>Plectin</i> ^{-/-} mouse	86

Note: MD = muscular dystrophy, CMD = congenital muscular dystrophy, LGMD = limb girdle muscular dystrophy, EB and MD = eye brain and muscle disease, XR = X-linked recessive, AD = autosomal dominant, AR = autosomal recessive, ^{-/-} = gene knockout.

Source: Modified from Durbeej, M. and Campbell, K.P., *Curr. Opin. Genet. Dev.*, 12, 349, 2002.

mutants e.g., the SJL mouse,⁷⁴ or by the generation of transgenic mice, in particular the production of specific gene knockouts. These are summarized in Table 10.2. The characterization and production of these animal models has enabled a much deeper understanding of the pathological processes underlying muscular dystrophy.^{87,88} A number of these genetically modified mice have also been used for testing therapeutic approaches for the corresponding human disease. For example the α -sarcoglycan knockout mouse has been used to demonstrate effective gene therapy using viral vectors⁸⁹ and stem cell transplantation.⁹⁰

MYOTONIC DYSTROPHY

The most common inherited adult myopathy is myotonic dystrophy (DM1), an autosomal dominant disorder. This condition is due to the expansion of a triplet (CTG) repeat in the 3' untranslated region of a protein kinase gene called DMPK. Core features of myotonic dystrophy are myotonia, muscle weakness, cataract, and cardiac conduction abnormalities.⁹¹ Myotonic dystrophy is an unusual disease in that it exhibits the phenomenon of anticipation. In the mildest form it is difficult to detect, and the only clear sign may be cataracts. However, the phenotype is more marked in the next generation, with a clear onset in early adulthood, with muscle weakness and

disorders in a number of other body systems. The offspring of affected individuals are in turn much more severely affected with evidence of congenital abnormalities. This phenomenon of anticipation, i.e., worsening disease with each generation, is associated with expansion of the triplet repeat.⁹² A similar but clinically separate condition, proximal myotonic myopathy (PROMM or DM2), was described more recently⁹³ and has been shown to be due to another repeat (CCTG) expansion of a gene located on chromosome 3q21,⁹⁴ whereas the DM1 mutation is found on chromosome 19q13. The great similarity between these two conditions has helped to convince the majority of those working in the field that most of the symptoms of the two diseases are likely to be due to the triplet repeats sequestering RNA-binding proteins, altering splicing and/or levels of messenger RNA (mRNA). However, differences between the two conditions suggest a possible role for loss of function of genes from the DM1 locus (DMWD, DMPK, and SIX5) to explain clinical aspects that are unique to DM1.⁹⁵

Transgenic mice have played an important role in understanding how the triplet repeat expansion causes the many different effects seen in myotonic dystrophy.⁹⁵ The effect of the repetitive element has been demonstrated in transgenic mice carrying a sequence of untranslated CTG repeats. These mice exhibit a myotonic phenotype⁹⁶ and abnormal splicing patterns in a number of gene products including CIC-1.⁹⁷ Most recently, disruption of the mouse Mbnl1 gene, an RNA-binding protein, has been shown to lead to muscle, eye, and RNA splicing abnormalities that are characteristic of DM1.⁹⁸ These results further support the hypothesis that manifestations of DM1 and DM2 can result from sequestration of specific RNA-binding proteins by a repetitive element expansion in the mutant RNA. Efforts are underway to develop a treatment for these conditions based on destruction of the mRNA produced by the mutant allele by administration of ribozymes.^{99,100}

ION CHANNEL MYOPATHIES

Myotonia

Becker syndrome, a recessive nondystrophic myotonia caused by mutations in the chloride channel 1 gene (CLCN1), is characterized by delayed muscle relaxation after contraction. The ADR (arrested development of righting response) mouse is an animal model for Becker syndrome.¹⁰¹ Skeletal muscles from ADR myotonic animals show an increased number of oxidative fibers with a lack of glycolytic fibers, as well as signs of muscle hypertrophy. The mto (adr^{mto}) and adr^k mice are allelic variants with the same clinical presentations but different mutations in the CIC-1 gene.¹⁰² All strains first show onset of myotonia at 10 to 12 days of age, and all have problems righting themselves after being placed on their backs, due to muscle rigidity caused by the myotonia. Growth rate is reduced compared to controls, but muscles show hypertrophy and a shift towards a more oxidative phenotype, presumably reflecting the increased activity associated with myotonia. The ADR mouse has been used to evaluate antimyotonic agents.¹⁰³

Thomsen's disease is another myotonia associated with mutations in CLCN1 and generally appears as a dominant condition but with less severe myotonia than Becker syndrome. The myotonic goat has long been recognized as a model of human myotonia. The goats start to develop myotonia at ages as young as 2 weeks and demonstrate muscle spasms in response to sudden movements. Sustained activity leads to more normal muscle activity, and the muscles of affected goats show few signs of pathological changes. The disease is inherited in an autosomal dominant manner and has been shown to be due to a point mutation in the chloride channel 1 gene.¹⁰⁴ Thus the goat is a good large animal functional and biochemical model of Thomsen's disease.

Congenital myotonia has also been observed in dogs. Farrow and Malik have provided a comprehensive clinical description of the disease in the chow chow breed, with onset as early as 6 weeks of age and with increasing clinical severity as the animals grow older.¹⁰⁵ Muscle stiffness can cause the dogs to collapse, and respiration can become restricted, although with exercise the

symptoms are reduced. There is dramatic hypertrophy of the skeletal muscle and marked variation in fiber size and mild muscle necrosis. The disease is inherited in an autosomal recessive manner, as is a similar condition in miniature schnauzer dogs. The latter have been shown to have defective CIC-1 channels.¹⁰⁶

Hyperkalaemic Periodic Paralysis

Another ion channel myopathy in humans, called hyperkalaemic periodic paralysis (hyperKPP), is due to mutations in the skeletal muscle sodium channel α -subunit. A very similar condition has been reported in American quarterhorses and appears to be inherited in an autosomal dominant manner. Clinical signs start with transient muscle spasms that spread to involve most of the skeletal muscles, and attacks can last between 15 min and 7 h. At other times the horses are normal and indeed look very fit, as the condition is associated with a heavily muscled physique. The myopathy is due to a single base change mutation that has been spread within the breed.¹⁰⁷ Thus the horse is an excellent genetic model of the human condition and to date is the only validated model available.

MALIGNANT HYPERTHERMIA

Malignant hyperthermia (MH) is a life-threatening condition involving muscle rigidity, a hyper-metabolic state, and hyperthermia. MH is triggered by a number of stresses, in particular anesthesia. The hyperthermia can be dramatic, reaching 45°C, and is due to a rapid increase in the metabolic rate of the skeletal muscle.

MH appears in several breeds of pig, is of considerable economic importance, and thus has been the subject of intensive investigation in order to eliminate this condition from commercial farming operations. It appears to have become widespread due to selection of animals with heavily muscled lean carcasses. Histological examination of the muscles prior to an attack shows normal morphology, but following an episode of MH there is extensive necrosis and hypercontraction of muscle fibers. Experiments have demonstrated that the resting level of calcium in muscles of MH-prone pigs is approximately four times normal and that this increases to about 20 times normal during an MH episode. This is associated with an increased affinity of the calcium-sensitive calcium channel for ryanodine.¹⁰⁸ In the human MH syndrome inheritance is autosomal dominant, whereas in pigs it is autosomal recessive. Genetic studies have shown that MH in all breeds of pig is linked to a single point mutation in the skeletal muscle ryanodine receptor, implying that all cases have their origin in a single founder animal.¹⁰⁹ At least some cases of human MH are linked to mutations in the skeletal muscle ryanodine receptor,¹¹⁰ making the pig a good animal model of the human condition.

MITOCHONDRIAL MYOPATHIES

Mutations in genes encoding mitochondrial proteins commonly lead to defects in oxidation–phosphorylation capacity and can affect multiple organ systems that depend on oxidative metabolism. These include brain, liver, and cardiac and skeletal muscle. It has been hard to develop animal models of mitochondrial myopathies, and there have been no convenient natural mutants described that have gone on to be used for laboratory studies. As mitochondria are inherited in the cytoplasm of the egg and carry their own genome that encodes many, although not all, of the mitochondrial proteins, it has been hard to target many of the mitochondrial genes through standard transgenic techniques.¹¹¹ An alternative approach has been the implantation of human myoblasts from patients with mitochondrial myopathies into the regenerating skeletal muscle of an irradiated severe combined immunodeficiency SCID mouse.¹¹²

In an attempt to create an animal model of tissue-specific mitochondrial disease, Graham and colleagues¹¹³ generated “knockout” mice deficient in the heart/muscle isoform of the adenine nucleotide translocator (Ant1). This resulted in the typical histological appearance of mitochondrial myopathies, ragged-red muscle fibers and a dramatic proliferation of mitochondria, together with marked exercise intolerance. Mitochondria isolated from mutant skeletal muscle exhibited a severe defect in coupled respiration; this was associated with an increased production of reactive oxygen species (ROS). In the heart the high ROS levels lead to increases in the accumulation of mtDNA rearrangements and further mitochondrial damage.¹¹⁴

Wredenberg and colleagues¹¹⁵ have recently generated another mouse model for mitochondrial myopathy by disrupting the gene for mitochondrial transcription factor A (Tfam) in skeletal muscle of the mouse. As predicted, these mice developed a myopathy with ragged-red muscle fibers, accumulation of mitochondria with an abnormal appearance, and progressively deteriorating respiratory chain function in skeletal muscle. Enzyme and image analyses demonstrated a substantial increase in mitochondrial mass in skeletal muscle of the knockout mice; this appeared to be sufficient to supply normal levels of adenosine triphosphate (ATP). However, muscle force was reduced, and the authors speculated that this might be due to other factors apart from the supply of ATP.

METABOLIC MYOPATHIES

Glycogen storage diseases are relatively rare human conditions, yet a number of useful animal models are available for glycogenosis types II, III, IV, and VII.

There is a range of animal models for glycogenosis type II (also known as Pompe’s disease). Shorthorn cattle have been described with an early-onset form at age 3 to 7 months and a late-onset form at 1 to 1.5 years, both inherited in an autosomal recessive manner.¹¹⁶ The latter is similar to the human childhood form, with the development of muscle weakness. All affected cattle show lysosomal storage of glycogen in skeletal, cardiac, and smooth muscle and in the neurons and have low levels of acid α -glucosidase (also known as acid-maltase) activity. Two additional acid α -glucosidase mutations have been identified in Brahman cattle.¹¹⁷ Another model of glycogenosis type II is found in the Japanese quail.¹¹⁸ The quail shows similar muscle weakness, lysosomal glycogen storage in the same tissues, and approximately 10% of normal levels of acid α -glucosidase activity. The quail has been proposed as a model of the adult-onset form in humans.¹¹⁹ A canine model has also been described in the Lapland dog.¹²⁰ Finally, two acid α -glucosidase knockout mouse models have also been generated, showing glycogen accumulations in skeletal muscle, heart, and liver^{121,122} and reduced capacity to generate force.¹²³ This model has been used to test gene therapy approaches to treatment of this condition.^{124–127}

Glycogenosis type III has been described in the German shepherd dog, which exhibits muscle weakness and exercise intolerance from an early age. The dogs have glycogen accumulation in the liver and in skeletal, cardiac, and smooth muscle and central nervous system (CNS) neurons.¹²⁸ The activity of the debranching enzyme, amylo-1,6-glucosidase, is substantially reduced in these dogs.¹²⁹ A similar condition has also been reported in American quarterhorse foals.¹³⁰

Glycogenosis type IV has been reported in Norwegian forest cats with muscle tremors and an unusual gait.¹³¹ The disease is progressive leading to severe muscle atrophy and loss of ambulation. Glycogen storage is seen in multiple tissues including skeletal, cardiac, and smooth muscle and in the central and peripheral neurons. Inheritance is autosomal recessive, and there are very low levels of branching enzyme activity.

McArdle disease (glycogenosis type V) is a common metabolic cause of exercise intolerance and recurrent myoglobinuria. The disease is due to biochemical defects of the muscle isoform of glycogen phosphorylase, and a number of spontaneous natural mutant animal models are available to study this condition. These include a bovine model that has a point mutation causing an amino acid change in glycogen myophosphorylase¹³² and a sheep model that has been demonstrated to have a deletion in the glycogen myophosphorylase gene.¹³³

INFLAMMATORY MYOPATHIES

Experimental autoimmune myositis (EAM) in animals can be triggered by injection of a homogenate of skeletal muscle and adjuvant and is used as a model of human inflammatory muscle disease. Myosin has been shown to be at least one of the antigens responsible for precipitating this autoimmune condition.¹³⁴ A recent study using a transgenic mouse overexpressing MHC 1 in the skeletal muscle has shown that autoimmune myopathies may arise as a consequence of an initial insult that allows presentation of autoantigens to the immune system.¹³⁵ High-dose intravenous immunoglobulin (IVIG) therapy has been effective in many autoimmune and systemic inflammatory diseases of humans including polymyositis (PM) and dermatomyositis (DM) and has been shown to be equally effective in the SJL EAM model.¹³⁶

TOXIC MYOPATHIES

A number of compounds that have been suspected of causing toxic myopathies have been tested in animals, and this approach has led to a better understanding of the mechanisms involved and how to avoid or reduce such problems in humans. The effects of a number of suspected myopathic toxins have been confirmed by administration to animals, for example zidovudine (AZT),¹³⁷ chloroquine,¹³⁸ and ethanol.¹³⁹ Some of the statins, hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, have been linked with skeletal myopathy. One statin, cerivastatin, was recently withdrawn from the market due to an unacceptably high incidence of rhabdomyolysis; this effect has been replicated in rats in order to try to understand the pathogenic mechanism(s). The authors were unable to demonstrate a clear cause but concluded that neither mitochondrial injury nor a decrease in muscle ubiquinone levels is the primary cause of skeletal myopathy in cerivastatin-dosed rats.¹⁴⁰

Early studies of myopathies in captive animals such as the Australian Rottneest quokka, a small marsupial, were useful in demonstrating the regenerative capacity of skeletal muscle.¹⁴¹ In the case of the quokka, the problem was traced to a deficiency of vitamin E leading to myopathy and muscle weakness.¹⁴² Supplementation of the diet with vitamin E restored muscle strength and demonstrated the potential to restore muscle function after addressing the underlying deficit. Likewise, selenium deficiency has also been shown to cause myopathy.¹⁴³

A range of biological toxins, for example snake venom, can cause dramatic muscle damage. In many cases the muscle fiber is destroyed but not the cells that are capable of muscle repair. These cells are often referred to as satellite cells, as they appear to arise from rare cells that lie under the basement membrane of the muscle fiber but have their own clearly defined cell membranes. The snake venom phospholipase, notexin, has been used to induce experimental muscle regeneration in animal models to study the process of muscle repair and to improve the grafting of donor cells in animal models of muscular dystrophy.^{144,145} A number of other compounds have similar properties, including local anesthetics such as bupivacaine, as well as calcium substitutes such as barium.^{146,147}

SUMMARY

A considerable range of naturally occurring animal models of myopathies can be used to understand the pathophysiology of the different conditions and to develop possible treatments. Some, such as the *mdx* mouse, have been used extensively for such purposes and have been extremely valuable in understanding the disease process and assessing experimental therapies. Where spontaneous mutant rodents are not available, they can be produced using techniques of genetic engineering. As treatments are developed in rodent models it will be important in many

cases to test such therapies in larger animal models before moving to clinical trials. The pool of such models is limited as the genetic engineering tools available for use in rodents are either technically more complicated or practically impossible in the larger animal species. It is therefore important that spontaneous large animal models are effectively characterized and retained.

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CHAPTER 11

Animal Models for Otolaryngological Disorders

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PART I: THE NOSE AND SINUSES

Douglas A. Ross and Jagdeep S. Hundal

More than 31 million Americans suffer from chronic rhinosinusitis (RS), making it one of the most frequently reported diseases in the National Health Interview Survey.¹ Billions of dollars are spent each year on treating RS both medically and surgically. The last century has brought an incredible change in our perception of this disease process, especially in terms of its etiology and surgical management. Both animal- and human-based research has contributed to our better understanding of RS. Animal models have the advantage of isolating and testing different variables that cannot be controlled in human studies.

Knowlton and McGregor² were probably the first to report the use of a dog model to study mucosal regeneration after complete mucosal stripping within the maxillary sinus. Other researchers,^{3–5} studying mucosal regeneration within the frontal sinus, observed that mucosa regenerates with significant scarring and cyst formation leading to poor drainage or ostial obstruction. Walsh,⁶ in a study using a canine model, stressed maintaining the functional integrity of the nasofrontal duct to allow postoperative healing to take place within the frontal sinus. The dog model has also been used to study the blood flow within the nasal mucosa⁷ as well as to study blood gas changes associated with nasal packing.⁸

The first report on experimentally induced sinusitis, in a rabbit model, can probably be attributed to Hilding.⁹ He performed antrostomies through an external approach in four different locations: at the natural ostium, adjacent to the ostium, along the floor of sinus, and as far as possible posterior from the ostium. He observed that antrostomies through or close to the natural ostium led to maximum sinusitis as compared to those away from the natural ostium.

Then followed an era during which research on frontal sinus became the center of focus. Ballenger and Ballenger¹⁰ described the use of a canine model to explore the concept of “vacuum headaches.” Their attempts to block the nasofrontal duct were not all that successful and their results were equivocal. It was Schenck and coworkers^{11,12} who described a successful canine model of frontal sinus disease, induced by nasofrontal duct blockage. They used this model to evaluate the pathogenesis of mucocoeles and postulated two distinct types of mucocoeles — “pressure” and “trauma” mucocoeles.¹³ Schenck¹⁴ then used the same model to examine various experimental and clinical factors involved in the failure of frontal osteoplastic operation. He concluded that fat obliteration with closure of the nasofrontal duct is probably more reliable than obliteration by osteoneogenesis. In 1976, using the canine model, Schenck and Rauchbach further postulated that development of chronic frontal sinus disease required interplay of at least three variables: the infectious insult, the response of the sinus mucosa, and the variable patency of the nasofrontal duct.¹⁵

In 1981, Maeyama et al.^{16,17} directed attention back to the maxillary sinus. They induced sinusitis in rabbits by injecting *Staphylococcus aureus* bacteria repeatedly into the maxillary sinus. Johansson et al.¹⁸ glued the natural ostium shut through an anterior antrostomy and then the next day injected an aliquot of pathogenic *Streptococcus pneumoniae* bacteria into the maxillary sinus, producing purulent sinusitis in 100% of animals. Kumlien et al. evaluated the sinus blood flow in a rabbit model under normal conditions¹⁹ and in the presence of experimentally induced sinusitis.²⁰ Other researchers from the same institute^{21,22} followed suit and also reported an increase in mucosal blood flow with sinusitis.

After success in inducing experimental sinusitis, researchers turned their attention to studying effects of sinusitis and exploring various etiological factors implicated in the pathophysiology of this disease. Johansson et al.²³ and Drettner et al.²⁴ first reported depletion of energy stores (ATP) with a buildup of lactic acid in the mucosa during infection. Westrin et al.²⁵ found evidence of an increased number of secretory epithelial cells and goblet cells while studying mucosubstance

histochemistry in the lagomorph animal model. The same laboratory also induced sinusitis with *Bacteroides fragilis*, leading to more severe and longer-lasting inflammation and development of systemic antibodies.²⁶ Using pneumococcal antigen, Westrin et al.²⁷ were also able to demonstrate correlation between antibody titer elevations and severity of infection. Later, the researchers at the Karolinska laboratory in Sweden studied sinomucosal histology under various conditions, using both light and electron microscopy, to address the etiology of sinusitis and polyposis.^{28–30}

At the same time, other researchers also developed interest in this animal model and began using it to further evaluate sinusitis. Min et al.³¹ observed that reopening the sinus ostia to establish air circulation decreased severity of infection. They also noticed that systemic and topical polymer delivery of antibiotics after ostial opening reduced the infection, and topical polymer particularly had some advantages.³² Topical nasal decongestants have been shown to worsen sinus infections. This has been attributed to decreased mucosal blood flow³³ and loss of cilia, with ciliary loss being more pronounced with the use of phenylephrine rather than with oxymetazoline.³⁴

The role of ostia in ventilation of sinuses is very critical, and Scharf et al.³⁵ demonstrated that occlusion of maxillary sinus ostia leads to the development of a negative pressure, reaching a subatmospheric plateau of 28.2 ± 7.3 mm of H₂O within 20 to 50 min a fact first quantitatively studied in a rabbit model. Similar changes had been earlier reported for the frontal sinus in cat³⁶ and dog models.¹⁰

Using a murine model, Blair et al.³⁷ concluded that a local allergic response created by sensitizing mice to ovalbumin augments bacterial sinusitis, underscoring the importance of allergic rhinitis in the development of sinusitis. Nitric oxide (NO) has been shown to be a regulator of mucociliary activity in the maxillary sinus mucosa,³⁸ and Schlosser et al.³⁹ have documented elevated levels of nitric oxide metabolites in chronic sinusitis rabbit models. These levels return to normal during recovery from sinusitis, implicating a possible link between NO and epithelial autotoxicity.

While the induction of sinusitis by occluding the maxillary ostium is accepted by many as the method of choice for creating sinusitis in animal models, some investigators oppose this method. Hinni et al.⁴⁰ criticized this model, suggesting that it violates the nasal mucosa and sets up an inflammatory reaction before inoculation of infection. They advocated a technique to close the ostium via an external approach through the nasal roof. According to Marks,⁴¹ however, both of these models are nonphysiologic and “sinogenic,” since these models involve only the maxillary sinuses and exclude ethmoid sinuses, which in humans are very important in the pathophysiology of sinusitis. Also, complete ostial obstruction in humans will lead to muco/mucopyocele formation and not sinusitis. Some investigators believe the most physiologic method for creating maxillary sinusitis is by inserting a foreign body (Merocel) into the nasal cavity and then impregnating it with pathogenic bacteria. The histologic analysis of sinusitis induced in such a manner revealed features typical to other known models of sinusitis and also showed a unique presence of discrete lymphoid aggregates. The presence of lymphoid aggregates, according to the authors, suggests the possible role of this model for evaluating the immune response of sinuses.⁴² A similar method of inducing chronic bacterial RS in a murine model has been described by Jacob et al.⁴³

Bomer et al.,⁴⁴ while acknowledging that the rabbit is the best-studied animal model for sinusitis in world literature, have suggested the use of a mouse model. The inability to manipulate rabbits genetically and lack of reagents and antibodies for detailed study of inflammation prompted them to develop a mouse model. They induced sinusitis by intranasal inoculation of *Streptococcus pneumoniae* and then studied histological sections to determine the percentage of neutrophilic infiltrate in mucosa and neutrophil clusters in the sinus cavity. The presence of infection in all animals, based on above-described criteria, made them conclude that this is a viable model for studying sinusitis and can be used to evaluate genetics and inflammation, even though surgical manipulation is limited. Ramdan et al.⁴⁵ have also used the murine model to study the paranasal sinus mucosa's histologic and immunologic response in viral rhinosinusitis. They found that for as long as 2 weeks after clearance of viral antigens, the immune response persisted at its heightened levels.

The management of sinusitis too has attracted the use of animal models, especially rabbits, to evaluate various medical and surgical modalities. Ingram et al.⁴⁶ have evaluated the role of mitomycin C, which has an antiproliferative action on fibroblasts, in rabbit sinus surgery. Their results suggest that the drug can be used to delay closure of antrostomies after sinus surgery. Similarly Sugiura et al.⁴⁷ have used the rabbit model to assess the safety of S-carboxymethylcysteine (S-CMC) in directly enhancing mucociliary activity in chronic sinusitis. They concluded that concentrations of 0.5 to 10% S-CMC are quite safe for nebulization when treating chronic sinusitis.

Over the years surgical concepts too have undergone a radical change, largely due to various studies performed in animal models. Because of Hilding's⁹ pioneering work, rhinologists started avoiding surgery on the natural ostium. Hilding demonstrated development of sinusitis in all animals in which the natural ostia were widened. Similarly the changes observed in regenerated mucosa following radical surgery,³⁻⁵ such as submucosal fibrotic proliferation, reduction in submucosal gland numbers, infiltration of inflammatory cells, and altered mucociliary transport,⁴⁸ have placed radical surgery into question and ushered in the concept of a conservative approach with minimally invasive endoscopic approach.

The surgical management of chronic frontal sinusitis too has been studied with the help of various animal models. In the early part of the twentieth century, frontal sinus obliteration was experimented with in dog and cat models using various surgical techniques and implants. Bregara and Otoiz⁴⁹ introduced adipose tissue for sinus obliteration, and later Montgomery and Pierce⁵⁰ popularized this technique. This technique had its limitations, however, such as requiring an additional abdominal procedure, increasing the risk of postoperative wound infections, fat necrosis with mucocele formation, and persistent frontal pain. This led Schenck et al.⁵¹ to use biomaterial (Proplast) in experimental studies. Unfortunately, Proplast evoked a strong foreign body reaction requiring frequent removal of the implants. Friedman et al.⁵² in 1991 reported use of hydroxyapatite cement for obliterating and reconstructing the cat frontal sinus. Nine cats were studied over 18 months with the opposite site serving as control; they had no adverse reactions, infection, mucocele, or implant extrusion. Recently, frontal sinus obliteration with heterogeneous cortico-cancellous bone and autogenous bone plugs were compared in monkeys. Both procedures were found to be effective, although obliteration with autogenous bone led to earlier obliteration.⁵³

A porcine model has also been used to study the effects of endoscopic sinus surgery on facial skeletal growth.⁵⁴ Investigators studied 30 pigs placed in five experimental groups:

1. Unilateral uncinectomy
2. Bilateral uncinectomy
3. Unilateral uncinectomy, anterior ethmoidectomy, maxillary antrotomy
4. Bilateral uncinectomy, anterior ethmoidectomy, maxillary antrotomy
5. Unoperated controls

Animals were killed after 3 months and growth assessed according to linear and spatial measurements of multiple craniofacial regions. The results showed significant restrictive shape alterations in a linear fashion in groups 1 through 4; the authors concluded that endoscopic sinus surgery causes significant restrictive effects on growing porcine facial skeleton.

In conclusion, animal models have played a very significant role in understanding the pathophysiology of sinusitis and in formulating various treatment modalities. The rabbit model has been demonstrated to be the most suitable model over the years for studying and managing sinusitis because the large size of the animal and presence of a single ostium, akin to humans, permit easy surgical manipulation. Also, sinusitis induction with help of an occluding nasal foreign body (Merocel) impregnated with pathogenic strains creates a sinusitis model best suited to study the pathophysiology of human sinusitis. If the aim is to study genetics and inflammation immunohistochemistry, however, a mouse model may be a better alternative.

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PART II: THE LARYNX

Clarence T. Sasaki and Jagdeep S. Hundal

The human larynx subserves three basic functions: (1) protective, (2) respiratory, and (3) phonatory. It comes at no surprise that each specific function of the human larynx is best represented by a specific group of animal models and that no single species is likely to accurately and precisely represent all of the functions governing the human larynx. The last century has witnessed tremendous progress in developing newer and better ways of treating patients. This progress and development are mainly attributed to an improved appreciation for the function of the human body. The quest for better understanding turned our attention to the human cadaver and specific animal models.

The noted French physician and anatomist Antoine Ferrein (1693–1769) probably first described use of an animal model to understand laryngeal physiology. His extensive investigations on excised human, dog, bull, pig, and sheep larynges revealed that vocal intensity depends on glottal width and air velocity.¹

Since then a variety of animal species has been used to study laryngeal physiology, including its phonatory function. The canine model has been extensively studied for this purpose due to its similarity to the human larynx.^{2–4} Similarly, researchers have used the feline model to investigate reflexes associated with protective glottic closure.^{5,6} Shaker,⁷ while studying the neural pathway and target organs of esophagoglottal closure reflex responses, concluded that while the cat demonstrated this reflex, its presence could not be ascertained in opossum and monkey. Again, no single model appeared globally representative of the diverse functions of the human larynx.

PROTECTIVE FUNCTION

Sasaki⁸ compared the presence of protective ipsilateral and crossed adductor reflex responses in cats, dogs, and humans. While the presence of ipsilateral adductor responses was observed with varying latency in all of the species, contralateral protective responses were consistently recorded in cats only, leading to the conclusion that the dog model is closer to the human and may be better suited to study protective neurophysiologic responses. Later, others demonstrated that the pig model had also been successfully used to study protective laryngeal reflexes, specifically demonstrating the presence of the cross reflex under varying levels of anesthesia,^{9,10} the presence of the laryngeal chemoreflex,¹¹ and protective esophagoglottal closure reflex.¹²

The implication of the laryngeal chemoreflex (LCR) in sudden infant death syndrome led to various investigations in a number of animal models.^{13–15} Such studies demonstrated the presence of liquid sensitive receptors in sheep, cat, monkey, pig, and dog, capable of evoking the LCR. The canine model has also been used to elucidate the age-dependent maturation of LCR,¹⁶ while the pig model was used to evaluate the effect of acetazolamide and intralaryngeal carbon dioxide on LCR.¹⁷ Again, Sasaki¹⁸ demonstrated in beagle pups that a transient period of adductor hyperexcitability could be identified 50 to 75 days postnatally and that the mechanism of the hyperexcitable state resulting in a transient

increased risk of laryngospasm appeared related to: (1) the completion of central synaptic maturation, (2) transient reduction in central latency, and (3) reduction in central inhibition.

RESPIRATORY FUNCTION

A heterogenous group of animals has also been used to evaluate the respiratory function of the larynx. Sasaki¹⁹ demonstrated changes in laryngeal abductor activity in response to varying ventilatory resistance in both the cat and dog model. In research at that laboratory, it was found that canine cricothyroid respiratory activity was directly modulated by ventilatory load and that receptors responsible for this modulation were likely located intrathoracically and mediated by the afferent vagus nerve.²⁰ On the other hand, Tsubone²¹ showed in anesthetized spontaneously breathing rabbits that respiratory modulation was due to changes in upper airway pressure, with positive pressure stimulating superior laryngeal nerve (SLN) afferent activity and negative pressure inhibiting it. They concluded that in rabbits the epiglottis was the main source of SLN afferent activity and that its displacement due to upper airway pressure changes was the most important factor for modulating SLN activity. Other investigators have used goats to study the SLN afferent activity under varying respiratory conditions, e.g., tracheotomy breathing (TB), upper airway breathing (UAB), tracheal occlusion (TO), and upper airway occlusion (UAO), as well as respiratory changes in response to transmural pressure applied to the larynx. They recorded increased activity within whole SLN at both inspiration and expiration due to TB, UAB, and TO, while UAO caused expiratory augmentation and inspiratory inhibition. However, the reflex response of upper airway muscles to laryngeal pressure changes in shiba goats was found to be less noticeable than in rabbits and dogs. In the guinea pig model ventilation was also shown to be affected by SLN section. In the intact SLN model, hypercapnia caused a concentration-dependent increase in respiratory frequency, tidal volume, and minute ventilation, but hypoxia had no effects. SLN section reduced respiratory frequency and minute ventilation during normoxia and reduced the ventilatory response to 6% CO₂.²²

Sant'Ambrogio²³ further demonstrated three types of laryngeal receptors in dogs: pressure receptors, flow receptors, and drive receptors, based on their responses to tracheotomy breathing, upper airway breathing, tracheal occlusion, and upper airway occlusion. Sixty-five percent of these receptors were active during eupneic breathing, and their activity increased markedly during upper airway obstruction resulting in an increased duration of inspiratory phasic abduction whereas bilateral SLN section abolished this effect. Because diverting airflow from tracheotomy to upper airway caused an increase in posterior cricoarytenoid PCA activity, these results supported the importance of SLN in regulating the pattern of breathing.²⁴ Using the swine model, Stella²⁵ also concluded that laryngeal afferents mainly from pressure receptors were influential in modulating the respiratory activity of laryngeal muscles.

PHONATION

The study of phonatory function has attracted the use of several animal models. Various vocalizing animals including dog, cat, pig, white-tailed deer, and monkey have been evaluated for this purpose. Kurita²⁶ observed that the thickness of the pig vocal fold mucosa (0.9 mm) is similar to that of the human (1.1 mm) as compared to the dog (3.3 mm). Also noted was the ratio of length of the vocal fold membranous portion to thickness of mucosa, which measured 20:1 in pigs, 14:1 in humans, and 5:1 in dogs. These findings along with the similarity in distribution and density of elastin and collagen fibers in the pig model allowed researchers to conclude that the pig larynx was a suitable phonatory model.

Jiang,²⁷ in a study evaluating laryngeal anatomy and function in four different species (human, pig, dog, and white-tailed deer), observed that although vocal fold lengths were similar among them, interaction of additional phonation-related structures differed. The human, pig, and dog larynges showed greater rotational mobility at the cricothyroid joint than the deer larynx did, whereas the thickness and structure of the vocal fold cover and vocal fold stiffness of the pig more closely resembled the human. The acoustic analysis of the vocal product among these species also revealed that the phonation range in pigs was closest to humans, leading to the conclusion that from a structural perspective, the pig's larynx was a superior phonatory model.

However, Garrett et al.²⁸ concluded that the dog was the most suitable animal model for surgical manipulation, specifically for benign nonneoplastic vocal fold lesions. They found that both dog and pig vocal folds had a similar microflap plane to that found in human vocal folds. However, the presence of glandular structures in the pig vocal fold, close to the medial vibrating edge, hindered the ability to elevate an extensive microflap in this animal model. This observation along with postoperative similarities found on laryngeal videostroboscopy supported the canine model for surgical dissection. Others have favored the canine model for evaluating phonatory dysfunction as a result of neurological lesions²⁹ and for evaluating the outcome of various procedures employed for treating them.³⁰

Thus, various animal models have been used over the last two centuries to study laryngeal functions as well as disease processes afflicting them. While in the early part of the previous century, the canine and feline models prevailed, newer regulations and stricter guidelines applied by animal care committees have created a shift toward animal models that are not commonly used as domestic pets. As a result, the pig larynx has been demonstrated to resemble the human larynx most closely in the support of investigations of protective and respiratory reflexes as well as phonatory functions.

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PART III: TASTE

Linda Bartoshuk

The sense of taste has similar functions across species. However, some quality-specific differences across species suggest that not all can provide good animal models for human taste. Taste identifies a small group of substances that are crucial to survival. Sweet and bitter tastes are produced by specific features on molecules that interact with complementary receptor sites. Sweetness is produced by only a few sugar molecules that are biologically useful to mammals: glucose (fuel of the brain), fructose (convertible to glucose in the liver), and sucrose (a disaccharide made up of a glucose and a fructose molecule). Similar structures not useful to mammalian physiology are tuned out. Artificial sweeteners produce variable responses across species. For example, aspartame (NutraSweet®) does not stimulate the same fibers stimulated by sucrose in lower species such as the hamster.¹

The bitter taste is believed to have evolved as a poison detector. Bitter systems across species are similar in that each species has multiple receptors that detect features of poisonous molecules.

However, these multiple receptors feed into a very few nerve fibers projecting to the central nervous system (CNS). All species need to be able to respond to poisons, but there is little reason to make them discriminable since the important response is rejection. The ability to taste specific bitter compounds varies across species (and even within members of a given species). This may be the result of evolutionary pressures operating to provide protection against poisons to a given species in a given environment.

The positive ions of electrolytes (salts and acids) act as the stimuli for salty and sour tastes. The salty taste identifies the cation sodium. Sodium is essential to nerve and muscle function and any losses of sodium must be corrected rapidly or animals die. NaCl appears to stimulate via two mechanisms: one involves movement of sodium ions through channels on the membrane of taste receptor cells and the other may involve diffusion of salt through the tight junctions between receptor cells.² The roles of these two mechanisms in human salty taste remains unclear.

The sour taste is something of a puzzle. Some have suggested that sourness could help primates reject unripe fruit. Sourness also protects against corrosive acids. The stimulus for sour is the passage of hydrogen ions through specific channels on the taste receptor membrane.³

ANATOMY

Taste buds are clusters of taste receptor cells usually found in papillae (the palate has taste buds not contained in papillae). The taste buds are buried in the tissue of the papillae and are connected to the tongue surface by openings called taste pores. Each taste bud is surrounded by nerve fibers thought to mediate pain/irritation.^{4,5} Each fungiform papilla is also innervated by touch fibers.⁶⁻⁸

The tongue is covered with small papillae in all mammals, although the structures of each papilla type vary across species.⁹ Fungiform papillae are found on the anterior tongue (innervated by the chorda tympani branch of cranial nerve [CN] VII). Foliate papillae are located on the edges of the rear of the tongue (often dually innervated by the chorda tympani and glossopharyngeal [CN IX] nerves). Circumvallate papillae form an inverted V on the rear of the tongue [innervated by CN IX]. Somatosensation (touch, pain, temperature) is mediated by the trigeminal nerve on the anterior tongue. On the posterior tongue, taste and somatosensation are both innervated by CN IX. Of special importance, interactions exist among the inputs of these nerves in the central nervous system. These interactions explain much that is known about taste disorders in human patients (see below).

Genetic variation for bitter substances occurs in many species and has been studied extensively in the mouse.^{10,11} Genetic variation has been known in humans since the discovery of taste blindness.¹² Links between genetic variation in taste and tongue anatomy were first discovered in the mouse; density of taste buds was shown to associate with magnitude of neural response.¹³ Research on genetic variation across species has focused on bitter compounds. In the mouse, the bitterness of sucroseoctaacetate is mediated by a single gene while the bitterness of 6-*n*-propylthiouracil (PROP) is mediated by multiple genes. That situation is reversed in humans. Since variation in the ability to taste PROP in humans affects food preferences and thus diet and health risks, PROP taste has been the subject of a considerable amount of research in humans (e.g., see^{14,15}). Humans can be divided into three groups: those who taste little bitterness from PROP (nontasters), those to whom PROP is moderately bitter (medium tasters), and those to whom PROP is intensely bitter (supertasters). Nontasters have the fewest taste buds; medium tasters, an intermediate number; and supertasters have the most taste buds.¹⁶ Supertasters not only perceive the most intense tastes but also the most intense oral burn (chilis) and oral touch.¹⁵ Currently, there is no animal model for human supertasting.

CODING OF TASTE QUALITY

Pfaffmann pioneered recordings from single taste fibers in the cat chorda tympani, working in the laboratory of Lord Adrian in Cambridge, England. He expected to find fibers specific to the four basic tastes: sweet, salty, sour, bitter. However, the fibers from the cat had very different response profiles. Virtually all responded to acid, a subset responded to quinine as well, and another subset responded to NaCl as well. This led Pfaffmann to propose the across-fiber-pattern theory.¹⁷ He argued that a response from a single fiber could not unambiguously code a quality. Thus the CNS must use the pattern of activation across a population of fibers to determine the nature of the stimulus.

As more species were studied, it became clear that the cat was not a good model. In particular, Taste fibers in other species can be placed into four categories based on the stimulus to which they respond most vigorously; Frank found sucrose-best fibers, NaCl-best fibers, acid-best fibers and quinine-best fibers.^{18,19} Assuming that each of these fiber types coded only the quality to which it showed the best response produced a labeled-line theory of taste coding. Pfaffmann ultimately embraced the labeled-line theory as a result of a behavioral phenomenon in squirrel monkeys.²⁰ Squirrel monkeys prefer sucrose to fructose but show greater neural responses to fructose than sucrose. Analysis of single taste fibers showed that sucrose-best fibers responded to both sucrose and fructose, but NaCl-best fibers responded more to fructose than to sucrose. Pfaffmann reasoned that the sucrose-best fibers were responsible for the sweet tastes of both sucrose and fructose. The NaCl-best fibers were responsible for a salty taste. Since fructose stimulated both the sucrose-best and NaCl-best fibers, it must taste sweet-salty to the monkey. Presumably the monkeys preferred the pure sweet taste of the sucrose to the sweet-salty taste of the fructose.

Taste mixtures played an important role in debates about which theory could account for human taste experience. Historically, pattern coding theories produce synthetic mixtures. That is, when two stimuli with distinct patterns are mixed, the resulting neural response must be a combination of the two patterns; the natures of the unmixed components would be lost in such a combination. This kind of mixture phenomenon is called synthesis. Color vision is an example of a synthetic modality; e.g., when red and green lights are mixed, we see yellow. When quality is coded by a labeled-line system, the nature of the components in a mixture can be preserved; a low note and a high note played on a piano can both be heard. Until the debate over coding, the taste system was thought to be analytic by most experts. However, the theorists who favored the pattern theory revisited the issue and claimed to have found evidence that taste mixtures are synthetic. They did this by presenting a variety of mixtures and asking subjects to describe them as “singular” or more than one. A number of their mixtures were described as “singular.” However, they neglected to consider the phenomenon of mixture suppression. Different qualities suppress one another in mixtures. Thus mixtures can seem “singular” when the weaker taste component is suppressed. The properties of taste mixtures have been determined with psychophysical studies in humans. Interestingly, elegant animal psychophysical experiments permit this conclusion to be tested in other species: the qualities of components in mixtures remain identifiable, suggesting analytic mixing in those species as well.²⁰

TASTE DISORDERS

The similarities across species make research with many species useful for the understanding of human taste disorders. Taste disorders fall into two categories: taste loss and taste phantoms (sensations in the absence of obvious stimulation); the proof that these disorders are related owes much to animal models. Nerve cuts in various species provide models of clinical taste loss. The most common animal model for these studies is the rodent. Halpern and Nelson²² found that damage to the chorda tympani nerve produced intensification of neural responses recorded from the ipsilateral medulla of rat. This led them to propose a very important principle of taste

neurophysiology; chorda tympani input normally inhibits input mediated by the glossopharyngeal nerve in the CNS. When the chorda tympani is damaged (or anesthetized), that inhibition ceases leading to intensification of responses in the CNS area receiving input from the glossopharyngeal nerve. This has the important function of maintaining constancy of taste sensations in the face of localized peripheral damage, but human observations suggest that this constancy comes at a cost.^{23–25} Many human patients who have sustained damage to the chorda tympani experience taste phantoms. We suspect that the release of inhibition generates erroneous signals in the CNS leading to these phantoms.

ORAL SENSORY PHANTOMS

Anatomical studies done in a variety of species suggest that taste inhibition has other functions as well. In primates, taste projects ipsilaterally from the periphery into the CNS, crosses the midline at the cortex and then sends descending paths, at least some of which are inhibitory.^{26,27} In other species some of the taste input crosses during the ascent of input from the periphery to the cortex, so care must be taken when using those species as models for central mechanisms of taste in humans. Taste appears to inhibit oral pain; this would serve to diminish oral pain during eating. This might have aided survival of animals that sustained oral injuries. However, in the human living in the modern era, this mechanism appears to be responsible for burning mouth syndrome (BMS). BMS consists of intense oral pain in the absence of visible pathology. Sensory evaluation of oral function in these patients reveals severe taste damage, particularly to the ability to taste bitter on the anterior tongue. In addition, the intensity of the pain of BMS is proportional to the density of fungiform papillae; that is, BMS is found in “supertasters,” individuals born with an unusually large number of taste buds.²⁸ This finding led to the conclusion that BMS is a sensory phantom akin to tinnitus or phantom limb. BMS may contribute to oral pain whenever the cause of the pain damages taste nerve fibers as well as tissue. For example, cancer patients suffering from mucositis may experience pain originating both from oral lesions and from the CNS.

Taste inhibition may function more generally to protect eating behavior.²⁹ Anatomical pathways suggest that taste might inhibit a variety of additional activities incompatible with eating (e.g., cough, hiccup, gag, nausea, vomiting). This is especially interesting in the context of human patients who have undergone therapies for cancer that likely have damaged taste. Work with animal models not only suggests the pathways involved but also provides information about the mechanism of inhibition that suggests therapies for human symptoms resulting from taste damage. Taste inhibition is mediated by the inhibitory neurotransmitter GABA.^{30–32} Thus one might expect GABA agonists to reverse the effects of taste damage. The GABA agonist klonopin reduces both oral pain phantoms (BMS) and the taste phantoms noted above.³³

Although work with animal models led to our understanding of taste and oral pain phantoms, we do not have a good animal model of these phantoms. Our primary tool for their study is the use of anesthesia of taste nerves in normal subjects.³⁴

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PART IV: ANIMAL RESEARCH IN PERIPHERAL AUDITORY PHYSIOLOGY

Joseph Santos-Sacchi

BACKGROUND

Cataloging the microanatomy of the auditory end organ provided an important start for understanding the physiology of the peripheral auditory system. These anatomical studies, conducted on a variety of species, began in earnest following the development of the compound light microscope in the 1700s. The detail in some of the original work of such pioneers as Alfonso Corti is remarkable (see Hawkins¹ and references within), but alas the microscopic structures were necessarily altered by the decay following extirpation and dissection. As we shall see, this decay had a major impact on our understanding of cochlear physiology as well. Whereas the development of fixatives proved a boon for anatomists, physiologists would not benefit directly but would belatedly profit from histologists' appreciation of the need to preserve the natural state.

The cochlea, we now know, is a remarkable electromechanical device. Prior to the discovery of animal electricity and means to measure it, only the mechanical components of cochlea function could be investigated. Thus, the microscopic structures in the inner ear were used to deduce the mechanical foundation of cochlea function; for example, Helmholtz postulated his resonance theory based on the changing length (hence mechanical properties) of radial basilar membrane fibers. Clearly, in mammals, the basilar membrane with its varying dimensions and stiffness would be a prime candidate for the basis of hearing. Indeed, von Békésy's studies² confirmed the importance of the basilar membrane's mechanical qualities by demonstrating tuned traveling waves evoked by basilar membrane disturbances. von Békésy used a variety of animal models, mainly the human temporal bone, but also mouse to elephant, to assess the sharpness of tuning in the cochlea, and while the data he obtained contributed immensely to our understanding of cochlea mechanics and won him the Nobel prize in 1961, unforeseen factors worked to stymie an understanding of the true sensitivity and tuning of the mammalian cochlea. These factors were the same that stymied early anatomists, namely the difficult-to-avoid decay of normal cellular structure and function after death. These problems, though acknowledged by von Békésy, were argued away, so that for many years auditory aficionados were led to believe that his data were gold. In fact, there were serious problems with his experiments, which when ultimately realized and solved would help elucidate the roles of the two types of hair cells, the inner and outer hair cells, that the early anatomists identified with the aid of improved fixation techniques. To improve on the work of von Békésy, models other than the human would be required.

WHY DO WE STUDY ANIMAL MODELS?

There are two main reasons for the study of peripheral auditory physiology, namely, the quest to understand and cure auditory dysfunction, and simply to understand how our and other animals'

ears work. Clearly, study based on the former rationale would benefit from direct investigations on humans. Unfortunately, though, immense difficulties are associated with human experimentation on the auditory periphery. These problems include limited number of specimens, inability to control for pathological effects, and the inevitable problem of tissue destruction caused by delays in the access to the cochlea (caused by dissection difficulties due to the dense temporal bone and delays in acquisition following death). Thus, for many reasons and regardless of the clinical vs. scientific rationales, animal models are best for studies on cochlear physiology. The major hurdle in rationalizing a particular animal model is its validity and generality. As far as providing insight to the human condition, most investigators believe that a range of small mammals, including cat, chinchilla, guinea pig, rat, and mouse, are appropriate. Of course, one or another species may prove better for particular experimental questions. In general, however, these small animals are particularly well suited since very nice access to the cochlea structures can be had. This enhanced accessibility (and heightened concern for better physiological status) has led directly to enhancements of the findings of von Bekesy.

BASILAR MEMBRANE TUNING

The data on basilar membrane movement that von Bekesy obtained indicated that the response of the basilar membrane was linear, i.e., the magnitude of the displacement response grew linearly with the stimulus level. By extrapolating down to threshold levels, it was argued that basilar membrane movements at the threshold of hearing were a fraction of an atom's width. Tuning of the basilar membrane was also observed to be not as good as psychophysical measures, and von Bekesy sought reasons beyond the basilar membrane to explain how we hear so well. With the aid of animal models, these notions would dramatically change.

Using the squirrel monkey, Rhode and colleagues^{3,4} first noticed that in some animals a nonlinear growth in basilar membrane motions occurred as stimulation levels were changed. Importantly, this compressive nonlinearity of the basilar membrane response was vulnerable to the animal's status. Only when extreme care was used to maintain a healthy preparation did the response remain nonlinear, otherwise it resembled the data of von Bekesy. We know now that tuning on the basilar membrane is far sharper than the data of von Bekesy showed, indicating that his human temporal bone preparation and his animal preparations were working in a passive, damaged mode. Of course, the new Mossbauer measurement tool that Rhode and colleagues used helped to record at lower stimulus levels than von Bekesy could using stroboscopic illumination. Today, it is clear that the sharp tuning found in the eighth nerve has its direct counterpart in basilar membrane motion.⁵ This turnaround in the description of basilar membrane tuning highlights the caveat that while animal models have the potential to provide valid data, care must be exercised in order to attain validity.

THE COCHLEA IS ELECTRIC

In the 1700s, electricians (as serious students of electricity were then called) often relied on themselves or acquaintances to serve as subjects. Indeed, Volta perceived a deafening blast as a result of self-inflicted aural electrocution.⁶ Despite this early indication, it would be nearly two centuries after the controversial discovery of animal electricity that the electrical nature of audition would be confirmed. The discovery had to wait for the invention of the vacuum tube and oscilloscope, but once in hand Wever and Bray⁷ demonstrated the "cochlear microphonic," an electrical response of the hair cells (which they mistakenly took as eighth nerve firings) measured from electrodes placed on a decerebrate cat's eighth nerve. The response mimicked the acoustic stimulus, and they confirmed this finding in a number of animal species, including turtles and insects.

The study of the electrical activity of the cochlea took off, and small mammals were perfect models since their cochleae were easily exposed for electrode implantations. Extracellular (from scala media and the perilymphatic scalae) electrical recordings were made of the resting (e.g., endolymphatic potential) and sound-evoked potentials (cochlea microphonic, summing potential, and even action potentials; see Dallos⁸), but eventually these were usurped by intracellular recording directly from hair cells^{9,10} after the high impedance electrode was devised. The clear correspondence of electrical activity within the auditory periphery with hearing capabilities would place this measure above that of histopathologic determination of auditory insult and recovery.

ANIMAL MODELS OF CLINICAL IMPORTANCE

The anatomical identification and characterization of cochlear dysfunction is time-consuming. The classic histological work on otopathology (see Schuknecht¹¹), while quite informative, could not be used with high efficiency, simply because the animal or human temporal bone had to be removed and processed after death. The use of electrical measures to determine auditory sensitivity, especially those that were noninvasive (e.g., auditory brainstem response [ABR], compound action potential [CAP]) permitted ongoing and quantitative studies on the effects of noise exposure, ototoxic drug exposure, and hazardous chemicals (e.g., see Henderson and coworkers¹²). As a direct result of animal experimentation, we would no longer have to experience devastation such as that caused by the first uses of the ototoxic antibiotic dihydrostreptomycin.¹³ Nevertheless, while animal models have immensely contributed towards our understanding of pathologies that afflict humans, we must remain cognizant of species-specific differences.

One of the hottest areas of current research is that of hair cell regeneration (see Warchol¹⁴). Mammalian hair cells do not regenerate following destruction, as do those of some lower vertebrates. Thus, unlike some tissues that can be interrogated via cell culture, mammalian inner ears must be harvested for each new experiment. Perhaps some day, if the key that controls hair cell production is found, hair cell cultures may reduce the need for animal sacrifice.

In the heyday of electron microscopy, transport of electron-dense markers into various compartments of the inner ear was studied. These studies included, for example, movements of molecules out of the vasculature and into cochlea scalae, thus identifying the blood-labyrinthine barriers^{15–17} and movements of molecules across the round window membrane into the scala tympani.¹⁸ The latter experiments contributed to the scientific basis for the currently popular clinical approach of intratympanic drug delivery, notably used to deliver gentamycin for control of intolerable vertigo. This type of approach heralds a new era where the otologist will successfully treat previously inaccessible structures of the inner ear and will ultimately do so with the new tools of molecular biology.

FROM MOLECULES TO EAR

Some very important advances in auditory physiology have been made in recent years using the techniques of molecular biology. Two of them are especially dear to my heart, namely, the determination that mutations of the connexon 26 gene result in nonsyndromic deafness¹⁹ and the identification of the protein responsible for the mechanical activity of the outer hair cell.²⁰

Connexons are proteins that form gap junction channels between adjacent cells, in the ear's case, allowing ionic and metabolic communication between supporting cells.²¹ These channels were proposed to aid in the removal of harmful extracellular potassium away from active hair cells,²² and this is a likely reason for the mutation's devastation of hearing.

The mammalian outer hair cell has long been known to dance wildly in response to electrical stimulation;^{23–25} this mechanical activity is believed to promote the sharp tuning and nonlinearity

that Rhode and colleagues found in the basilar membrane. Finally, after nearly two decades, the motor responsible for the outer hair cell (OHC) boogie was molecularly identified as the protein prestin.²⁰ The story is a continuing one that is full of twists,²⁶ but most recently a knockout of the prestin gene in the mouse seriously interfered with normal auditory function,²⁷ and a mutation of the gene was shown to cause deafness in humans.²⁸ In the end, it will likely be the mouse model that holds the key to our interests in the ear, as this small prolific rodent is a perfect molecular biology laboratory. But let us not forget the guinea pig, which is a classic model in auditory research; indeed, this animal has just helped show that gene transfection (of Math1 transcription factor) into the intact cochlea can induce new hair cell growth.²⁹ Imagine, the future otologist growing some new hair cells for us hard-of-hearing baby boomers — thanks to animal research.

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PART V: ANIMAL MODELS OF HEARING LOSS — THE CENTRAL AUDITORY SYSTEM

Ilsa R. Schwartz

The recent very rapid increase in our knowledge of human deafness and hearing loss has only been possible because of the many studies of animal models of hearing that have gone before. Classical studies of the auditory system have identified all of its component parts, their structures, their basic functions, important aspects of their chemistry, and the changes in those components caused by genetic factors and by various pathologies. The vast majority of these studies have used animal models.

Classical models of human hearing loss have been other mammalian systems. Basic anatomical studies of the normal auditory system were carried out in a wide variety of mammalian species including the cat, dog, chinchilla, rabbit, ferret, rat, mouse, guinea pig, gerbil, and hamster. These studies provided the basis for understanding the structure of the component nuclei, their neurons, their synaptic connections, and the pathways from the ear to the cortex. In recent years, these studies have provided important information about neuronal changes in anatomy, chemical content, and connectivity during normal as well as pathological development and aging. A wide range of techniques has been employed in studies that identified and characterized the various central auditory structures (the cochlear nuclei, the trapezoid body, the superior olivary complex, the lateral lemniscus, the inferior and superior colliculi, the medial geniculate, and the cortex). Techniques employed range from very basic methods, such as Nissl and Golgi staining of neurons, to track tracing methods involving lesions and silver staining of degenerating neurons and mapping the course of retrograde and anterograde tracers injected into small areas of the brain or into individual neurons, to autoradiographic and immunocytochemical localization of various enzymes, structural proteins, and proteins associated with neurotransmitters. The wealth of information developed by these investigations is far too extensive to be reviewed here and the reader is referred to some of the excellent reviews (books, chapters, and papers) that cover all of the areas of the central auditory (e.g., Webster et al.¹). It is important to remember that practical diagnostic tools such as the auditory

brain response (ABR) could not have been developed and validated without animal models in which to perform lesion studies to identify the underlying structures giving rise to the various waves.

Basic physiological studies of the auditory system began with the cat and dog because of the greater ease of recording from larger structures but have also utilized a wide variety of other animal models including the chinchilla, ferret, rat, mouse, guinea pig, gerbil, and hamster. These studies characterized the basic properties of different groups of auditory neurons in the brain and laid the foundation for understanding normal and pathological states. These studies too have been extensively reviewed in recent books, papers, and chapters related to each level of the auditory system (e.g., Webster et al.²).

From the earliest observations that deafness and hearing loss ran in families, there has been recognition of a strong genetic component in many types of human hearing loss: congenital, progressive, and adult onset. Approximately 1 in 1000 babies is born with hearing loss, nearly 60% attributable to a genetic cause and the remainder to infectious or environmental exposures.³ In the United States, about 23% of those between 65 and 75 years of age and 40% of those over 75 have age-related hearing loss, known as presbycusis.⁴ We know that age-related hearing loss also has a significant genetic component.

The association between human deafness or severe hearing loss and pigmentation anomalies such as Waardenburg's syndrome led to the early use of animal models with pigment anomalies: the deaf white cat, the Dalmatian dog, and a number of mouse strains with white spotting or other coat color anomalies. While early studies focused on characterizing the changes in the structure and function of the ear and central auditory neurons in these genetically altered systems, progress in identifying the chromosomal location of the defects and of identifying the actual genes and gene products was gradual, progressing steadily with the development of tools for DNA analysis and the sophisticated study of linkage using DNA markers, culminating in the Human Genome Project. The explosive growth of genetic information about deafness and hearing impairment in the past few years has only increased the need for animal models. In less than 5 years from the end of 1996, when no human nonsyndromic deafness genes had been cloned, 19 new genes involved in nonsyndromic deafness were identified, together with an even larger number implicated in syndromic deafness.⁵ Of the 60% of human hearing loss cases that are of genetic etiology, about 30% are syndromic, about 56% are nonsyndromic with an autosomal recessive pattern of inheritance (DFNB), about 12% are nonsyndromic autosomal dominant (DFNA), and about 2% are nonsyndromic and either X-linked (DFN) or mitochondrial.³ As of 2002, more than 77 loci have been mapped with 50 auditory genes sequenced including 14 autosomal dominant, nine autosomal recessive, two X-linked, and five mitochondrial, plus at least 31 genes for syndromic hearing loss.^{3,6} The rapid increase in information about the human genome in recent years has made the mouse an even more important model system for the study of many auditory pathologies, with genes identified in the mouse being sought, and found, in human deafness and hearing loss. The number of different mouse genes affecting inner-ear development or function that had been identified exceeded 90 in 2001, with many more loci known to be involved in deafness but not yet identified and still more identified that affect the middle ear or central auditory pathways. With the cascade of new information, the most up-to-date listing of genes affecting hearing is available on the Web at sites maintained by the Jackson Laboratory (<http://www.jax.org/hmr/models.html>) and the Medical Research Council (<http://www.ihf.mrc.ac.uk/hereditary/MutantsTable.doc>). There is still a very limited overlap in deafness genes identified in mice and humans, indicating that many deafness genes remain to be found in both species. As human genes for hearing loss have been identified, animal models have been sought and used to investigate their molecular mechanisms. In this regard not only the mouse, but rat and zebra fish, and even *Drosophila*, have provided important models and investigative tools.

Although many of the recent genetic findings address genes that have their primary effect in the cochlea, a number of genes that have an effect on the central auditory system have also been identified (<http://www.ihf.mrc.ac.uk/hereditary/MutantsTable.doc>). Animal models that express the

mutant alleles of these genes are critical for studying changes caused by the mutations over time and under controlled environmental conditions. Further, once the mechanism of action of the identified genes is understood, animal models are critical to testing potential medical interventions. Determinations of the ototoxic effect of drugs, evaluation of factors contributing to ototoxicity, and potential methods for preventing or remediating the ototoxic effect are dependent on animal models.

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CHAPTER 12

Animal Models of Lung Physiology during Anesthesia

Göran Hedenstierna, Görel Nyman, and Claes Frostell

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INTRODUCTION AND ETHICAL CONSIDERATIONS

Animals are frequently used for the study of respiratory function, and many of these studies are conducted during anesthesia because the aim of the study is anesthesia per se, or because the execution of the study requires that the animal is anesthetized. The major focus of this chapter is to describe animal models that might be used for the study of lung physiology during anesthesia. Since there is a qualitative similarity, but a large quantitative difference, between anesthesia-related effects and those that can be seen in the intensive care setting, i.e., acute lung injury (ALI) or acute respiratory distress syndrome (ARDS), this will be described briefly. The chapter may also contribute some insight into pulmonary physiology that can be applied by veterinarians in their daily clinical work.

The ability to use animals for research rests upon an acceptance in society of the ethics of this practice. It is beyond the scope of this chapter to seriously discuss in detail this important issue. However, it deserves to be mentioned that we as authors strongly believe in the necessity to provide full-scale large animal research capacity to test any development of pharmaceutical or technical significance before going to human studies. There are enough physiological and pathophysiological similarities between humans and several species to justify this endeavor. Therefore, it is of utmost importance that animal experimentation is carried out in a transparent, controlled, educated, and highly ethical manner in which the integrity of these beings is respected as far as possible. This implies that there should be solid potential benefits from planned experiments and that the welfare of the animals is respected and a minimum of pain is experienced. Observing these points have made us to describe herein only experimentation that is to be performed during anesthesia and that end with the animal being killed by an overdose of anesthetics without ever being allowed to wake up. It is the rare experiment promising unusual benefits not to be reached otherwise that shall require animals to wake up attached to devices or catheters restricting their movements or feeding or that induce pain.

RESPIRATORY PHYSIOLOGY IN HUMANS — A BRIEF OVERVIEW

As a basis for comparative physiology, it might be appropriate to briefly review human pulmonary function. This will be done in the next few paragraphs.

Normally, we breathe more with the lower lung regions than with the upper ones, i.e., caudal regions, near the diaphragm in the upright subject, dorsal regions in the supine subject, and in the lower lung in the lateral position. This is the effect of a vertical pleural pressure gradient and a curved pressure–volume relationship. Upper lung regions are exposed to a more negative pleural pressure than lower ones are and are therefore more expanded than lower regions. Moreover, due to the shape of the pressure–volume curve there will be smaller volume increase in upper lung regions for a given pressure rise. Thus, lower lung regions receive more of the tidal volume.¹ This is illustrated in Figure 12.1. With increasing age we lose elastic tissue; the skin wrinkles and the airways are more flaccid. In the elderly, the airways may close during a normal expiration and they may open up during next inspiration.² In the worst case, airways will be continuously closed throughout the breath. Since the regional lung volume is smaller in dependent regions, airway closure will begin in the bottom of the lung, in dorsal regions if supine and in caudal regions if upright. In the lateral position, the lower lung will suffer from airway closure and the upper will

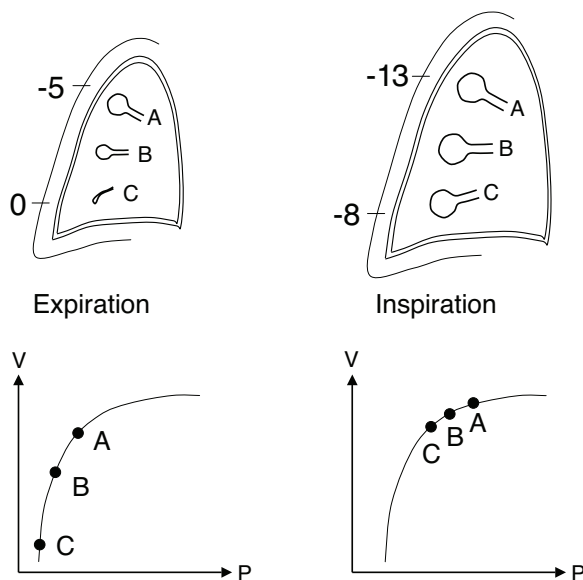


Figure 12.1 Pressure (P)–volume (V) curves of the lung. Letter A denotes an alveolus in the upper lung region, B in middle, and C in the lower, dependent regions. Note the different locations of A, B, C on the P-V curve at end-expiration and end-inspiration. The three alveoli follow the P-V curve with the same pressure difference between them both at expiration and inspiration. However, due to the shape of the P-V curve, their large differences in volume at expiration will decrease when they are inflated during inspiration. The largest volume change occurs in the lowest region C. Note the pleural pressure that is indicated in the schematic lung drawing in the upper panels. There is a difference between upper and lower regions; the upper is exposed to more negative pressure. During spontaneous inspiration, the pleural pressure becomes even more negative.

be spared. Thus, airway closure diverts ventilation away from lower regions. This is different from the younger healthy subject with no airway closure whose ventilation goes mainly to lower lung regions.

Not only ventilation but also lung blood flow increases down the lung so that the lower regions are better perfused than upper ones. The gravitational orientation of lung blood flow occurs because arterial pressure is lower in the upper lung regions than in the lower ones; the difference is explained by rising hydrostatic pressure down the lung.³

The relatively similar vertical distributions of ventilation and blood flow (Figure 12.2) ensure good matching between them and optimum gas exchange with an arterial oxygen tension (PaO_2) of around 10 to 13 kPa (75 to 100 mmHg) with a magnitude of ventilation that causes an arterial carbon dioxide tension (PaCO_2) of around 5.5 kPa (40 mmHg).

Almost all anesthetic agents (halogenated compounds, neuroleptic agents, barbiturates, and derivatives), both inhalational and intravenous, reduce respiratory muscle tone. This causes a shift in the balance between the outward forces of the respiratory muscles and the inward forces caused by the elastic recoil of the lung tissue, so that functional residual capacity (FRC) is reduced by approximately 0.4 to 0.5 liters⁴ in adult humans. The change from upright to supine causes a lowering of FRC by 0.7 to 0.8 liters. The net effect of change in body position and anesthesia is a decrease in FRC from an average of 3.4 to 2.2 liters (example from a 75 kg, 180 cm tall man). The fall is close to what can be reached during maximum expiration, the so-called residual volume. Breathing at this lung volume is highly uncomfortable, as can be tested by expiring maximally and attempting to breathe at a low lung volume (a demanding exercise test for the reader).

Not only will airways close in the anesthetized subject, alveoli may also collapse; this is called atelectasis.⁵ Atelectasis will occur in the bottommost lung regions and will comprise 10 to 15% of the total lung tissue in the average subject (Figure 12.3). It may exceed 30 to 40% of the lung in

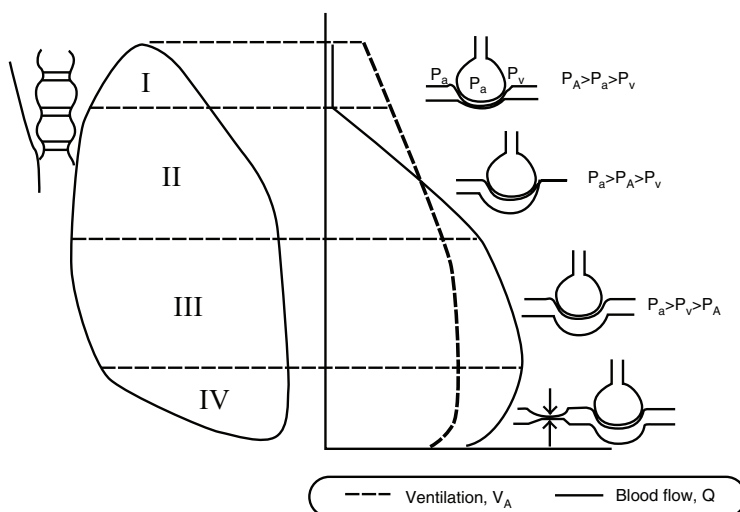


Figure 12.2 Distribution of ventilation and blood flow in the lung from top to bottom. Note the increase in ventilation down the lung and the even greater increase in perfusion. Zones I, II, III, IV are defined by the relationship of alveolar P_A , arterial P_a , and venous pressures as indicated in the right part of the figure.

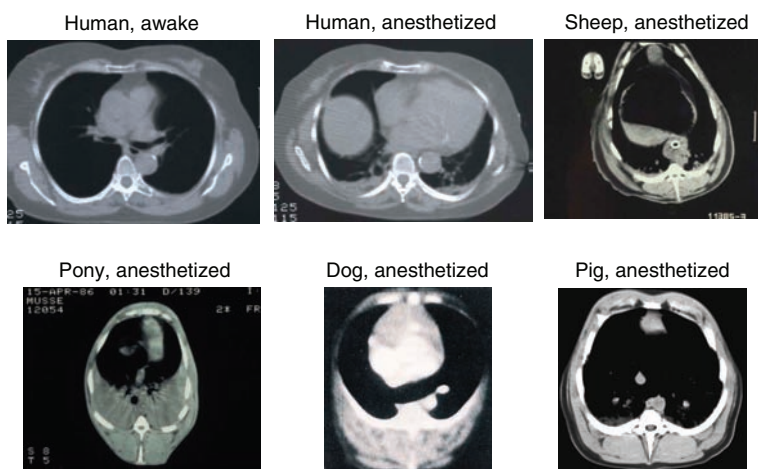


Figure 12.3 Transverse CT-scans of the chest in a human awake and during anesthesia and in four animals during anesthesia. Note the well-aerated human lung awake and the appearance of atelectasis in bottom of both lungs during anesthesia (seen as gray areas in the most dependent regions). The gray area in the middle area of the right lung (seen to the left on the CT-scan) is the diaphragm and the liver that have been moved cranially during anesthesia (the cut through the chest is the same awake and during anesthesia). As can be seen, the dog does not show any atelectasis, whereas sheep and pig show minor atelectasis. Many animals show no atelectasis. Finally, the pony shows huge atelectasis, covering more than half of the transverse thoracic area. Since atelectasis by definition is airless, there is considerably more tissue in the atelectatic area than in the aerated regions. Thus, more than two thirds of the lung has collapsed in the pony.

what may still be considered an uneventful anesthesia. However, it will cause considerable impairment of oxygenation of blood, as will be discussed below. There are two prerequisites for atelectasis to develop during anesthesia:

1. Loss of respiratory muscle tone with subsequent fall in lung volume, promoting airway closure
2. Ventilation with highly adsorbable gas (as oxygen) that will easily be taken up by the blood flow so that the gas pocket behind the closed airway is emptied⁵

Thus, ventilation with 100% O₂ causes atelectasis in 5 to 7 minutes whereas air (the major component (79%) of which is the poorly soluble gas N₂) takes several hours to adsorb and to cause lung collapse.⁶ It has indeed been shown that atelectasis in the anesthetized subject occurs during the induction of anesthesia when preoxygenation is provided. If preoxygenation is performed with 80% O₂ (makeup gas N₂), much less atelectasis is produced.⁷

MORPHOLOGICAL CHARACTERISTICS OF THE LUNGS AND THE CHEST WALL IN DIFFERENT ANIMALS

Thorax

The thoracic cavity and lungs differ between animal species both in anatomy and function. It is well known that dogs and cats are adapted to rest or sleep in a sternal, lateral, and even in a supine position whereas larger animals, such as cattle and horses, never sleep on their backs. Since prey animals must stay vigilant to survive, they tend to spend only a minor part of their time recumbent. Adapted to flight rather than to fighting, horses are able to sleep in a standing position due to the “stay apparatus,” a locking mechanism of the limbs. In ruminants, the continuous regurgitation of ingesta and gas from the rumen may commence in both standing and sternal positions, while gas is trapped when the animal is positioned in a lateral or supine position during anesthesia. To prevent bloat, resulting in concomitant impairment in ventilation and pulmonary gas exchange, excess gas needs to be evacuated from the rumen during anesthesia.

The respiratory system has functions besides alveolar gas exchange in both dogs and cats. In the dog, panting provides cooling of the body by evaporation of water from the nasal mucosa but increases dead space ventilation.⁸ Panting occurs during exercise but also in the resting animal at high ambient temperatures and may occur during anesthesia. Cats purr when they are content but may also do so when they are sick or sleeping. The sound is produced when diaphragmatic contraction alternates with glottal opening and closure. It is present both during inspiration and expiration. Purring may improve ventilation and prevent atelectasis during shallow breathing.⁹

Diaphragm

Large animals and some athletic dog breeds have a narrow thorax with a large vertical lung distance. In addition, the diaphragm slopes diagonally downward and forward so that the lungs lie on top of the abdominal cavity when the animal is standing. This anatomical arrangement, with the largest part of the lungs situated in the dorsal part of the thorax with little lung tissue lateral to the heart, may be a functional adaptation in athletic species (Figure 12.4). Turning these animals upside down during anesthesia will, however, reverse the favorable situation, causing the lungs to be squeezed down under the weight of the abdominal viscera.¹⁰

The tracheal dimension in relation to body size also varies among species. In flight animals, a high flow of air through the airways is efficient for improved ventilation during strenuous exercise. The cost of a wider trachea is larger dead space ventilation. Dead space ventilation in a giraffe is actually smaller than in a horse, probably due to the narrower but longer trachea.¹¹

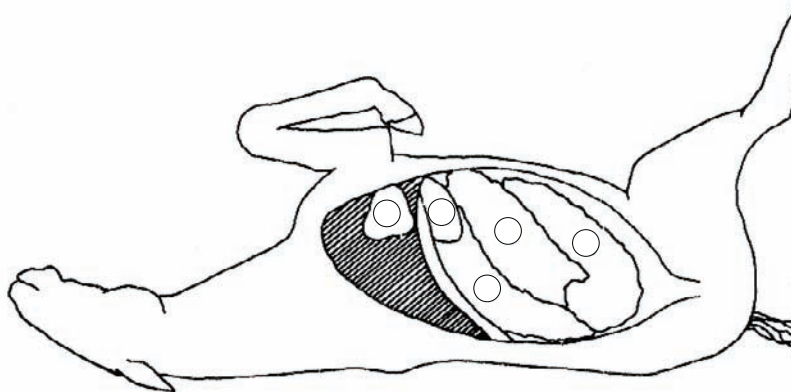


Figure 12.4 Illustration of the slanting diaphragm in the horse. In dorsal recumbency, the abdominal organs rest on the diaphragm, exposing the dorsal regions of the lung to considerable pressure. This contributes to the widespread atelectasis in the anesthetized horse.

Lung Lobulation

The mammalian lung can be divided into three types depending on lobulation, pleura, peripheral airways, and vascularisation.¹² Cattle, pigs, and sheep have lobulated lungs with terminal bronchioles and few respiratory bronchioles. They have thick pleura, and the pulmonary veins follow the pulmonary artery and bronchus in the peripheral lung. Both cattle and pigs have thick smooth muscle layers in the small pulmonary arteries.¹³ In dogs, cats, and monkeys, the lungs are not lobulated, the pleura is thin, and respiratory bronchioles are well developed. The lung vein runs separately from the pulmonary artery and bronchus. The smooth muscle layer in the small pulmonary arteries is thin in the dog.¹³ The horse differs from the previously mentioned species. It has incomplete septa between lobules, thick pleura, and poorly developed respiratory bronchioles. The vessels follow the bronchus in the periphery. The alveolar surface area of the lung and the maximal oxygen uptake are greater in the athletic horse and dog than in less athletic mammals.^{14,15}

Collateral Ventilation

Collateral ventilation means that the alveoli can receive inspired air not only via the major bronchi and bronchioles but also by communications between alveoli, so called Cohn's pores, and via interbronchiolar channels. The benefit of these communications is that if an airway is occluded by a mucus plug, an inflammatory or edematous airway wall, or by a foreign body, ventilation can be maintained to a certain degree in the otherwise closed-off region. This collateral ventilation will contribute to gas exchange between the alveoli behind the airway occlusion and lung capillaries (Figure 12.5). However, since air that is entering alveoli via these collaterals may have participated in gas exchange in other alveoli before, the quality of the air is secondary, with less oxygen and more carbon dioxide than room air. However, it will still be an advantage as compared to having no possibility of collateral ventilation. This will thus give the cat, rabbit, and dog an edge compared to the pig and cow.

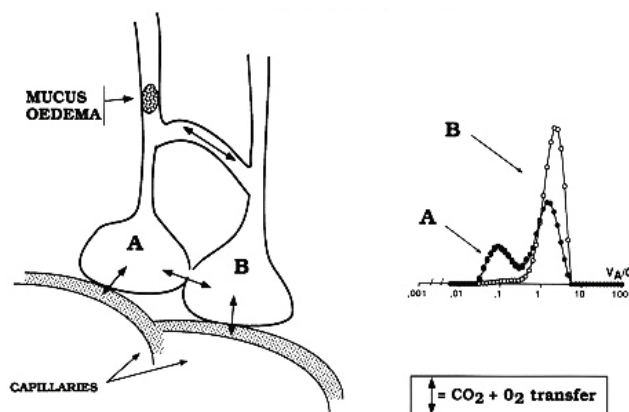


Figure 12.5 Illustration of collateral ventilation via Cohn's pores between alveoli and via interbronchial channels. This enables ventilation of otherwise closed-off lung units (e.g., mucus, edema, constriction, and foreign objects). However, the ventilation in region A is performed with gas that has already been participating in gas exchange in region B. This results in a bimodal distribution of the \dot{V}_A / \dot{Q} ratios (see also the paragraph on gas exchange, multicompartment model).

Hypoxic Pulmonary Vasoconstriction

Although different animal species use different mechanisms to match their ventilation and perfusion, either by collateral ventilation or pulmonary hypoxic vasoconstriction, or a mix of both, healthy animals achieve the same result. The strategy is probably related to the differences in lung structure and function. Cattle and pigs have no collateral ventilation and are dependent on redistribution of blood flow by pulmonary vasoconstriction. Dogs, cats, and sheep depend on collateral ventilation with little contribution from hypoxic vasoconstriction, whereas the horse primarily depends on hypoxic vasoconstriction with only a small contribution from collateral ventilation. Pulmonary hypertension in response to hypoxemia from exposure to high altitude is species dependent. Cattle can develop right heart failure and edema, "brisket disease," caused by chronic hypoxic vasoconstriction. The pulmonary circulation of sheep, llamas, and horses, grazing or exercising at the same altitude, is hyporesponsive to hypoxemia.^{16,17}

Since the pig is a frequently used research animal, it might be worth knowing that the pulmonary vasculature is also very sensitive to agents that irritate the vessels. These can be dextran given to prevent clotting but causing by itself vasoconstriction, microspheres for the measurement of blood flow distribution, or vasoactive drugs that will cause stronger responses in the pig than in other species.¹⁸

ATELECTASIS

While very little is known about airway closure in different animals, considerable experience has been gathered regarding atelectasis formation in different species during anesthesia.

Many early studies on the influence of anesthesia on respiratory function were done in dogs. However, the dog does not develop atelectasis during anesthesia as assessed by computed tomography (CT)-scanning¹⁹ and they develop less impairment of gas exchange. There is thus a fundamental difference as compared to the human. The cause of the difference has not been clearly shown but may be related to the thoracic shape, absence of a fall in FRC, and collateral ventilation. The latter would prevent or limit the formation of adsorption atelectasis.

Presently, most “large” animal studies are conducted in pigs (2 to 3 months old with a weight of 25 to 30 kg) and sheep. Both species develop minor atelectasis.^{20,21} Examples of atelectasis in different species are given in Figure 12.3. However, inflation of the stomach by air in the sheep produces larger atelectasis; the likely effect is decrease of lung volume and possibly compression of lung tissue and the adsorption of gas behind narrowed and closed airways. Thus, a model to produce atelectasis similar to that in humans during anesthesia might be to inflate the stomach in the anesthetized sheep with air.

Radiographic studies in horses showed that diffuse radiographic opacities developed in the lower lung in the laterally recumbent horse within 20 min after induction of anesthesia.²² These opacities persisted for some time when the horse was turned over to the other side. At the same time, opacities developed in the previously upper lung. Attempts to reexpand what was assumed to be collapsed alveoli by spontaneous deep breaths or forced ventilation failed to reduce the opacity in the dependent lung. In anesthetized Shetland ponies in dorsal recumbency, densities in dependent lung regions were observed on CT.¹⁰ The densities were similar to what has been seen in anesthetized humans, but they were more extensive in the ponies. Autopsy and microscopy of rapidly frozen lung tissue show that the densities are atelectasis. When turning one pony from dorsal to sternal recumbency, the atelectasis remained for some time where it had been created but was immediately diminished by two large volume insufflations. This is similar to what can be seen in anesthetized humans (Figure 12.6).

A cranial displacement of the diaphragm has been shown in the horse during inhalation anesthesia and spontaneous breathing and a concomitant decrease in FRC to about 50% of the value in standing horse.^{22,23} According to Sorenson and Robinson,²³ closing capacity exceeds FRC in dorsal recumbency, and they suggested that the loss of ventilated lung volume probably was due to compression of the lung by the weight of the thoracic and abdominal viscera.

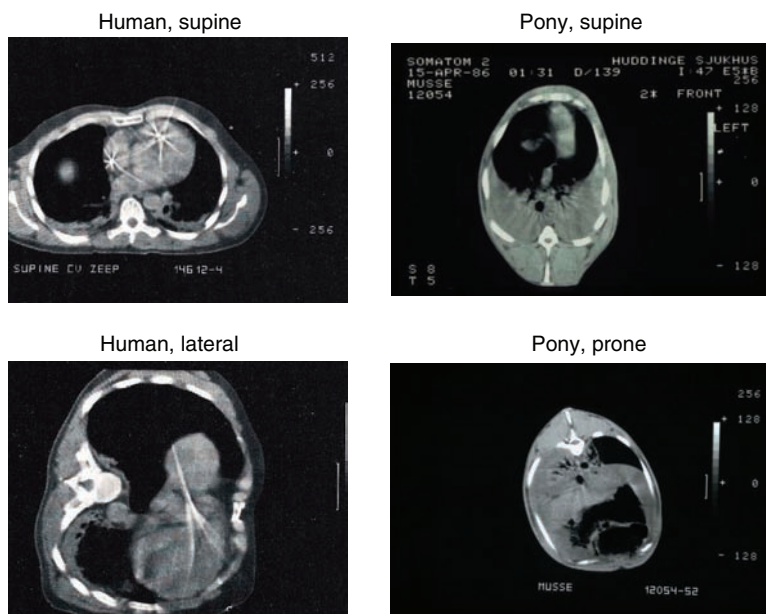


Figure 12.6 The influence of body position on atelectasis distribution in anesthetized human and pony. Note the atelectasis in dependent lung regions both in human and horse, and the maintenance and new formation of atelectasis in the dependent lung when the subject has been turned to the lateral position. The upper lung has less atelectasis, an effect of the increased volume of the nondependent lung. In the pony, some of the previously dependent region has been re-aerated in the prone position.

Acute Lung Injury

In acute lung injury or ARDS in the human patient, widespread collapse and consolidation of lung can be seen. Thus, CT scans show qualitatively similar changes as the anesthetized subject but quantitatively they are much larger (Figure 12.7). Initially, it was thought that the reason for airlessness was that the edematous lung caused collapse of lower lung regions by the increased “superimposed” pressure, making them atelectatic. There is also some evidence that the lung dimensions are maintained despite loss of air in a canine oleic acid lung damage model. This was demonstrated by implanting radioopaque steel beads in the lung and measuring the distances between them by radiography.²⁴ The maintained chest dimensions are explained by edema and free fluid formation that replace the air in airways and alveolar spaces. Another support for consolidation rather than atelectasis is that other animal models of either endotoxin sepsis or oleic acid edema show increased lung dimensions.²⁵ So whether atelectasis has occurred or not, consolidation by fluid accumulation must have been the dominating mechanism of airlessness.

This may be considered somewhat academic and of little clinical significance since both conditions will produce shunt and hypoxemia. However, the mechanisms behind the airlessness are of importance in understanding the process of acute lung injury and in developing techniques to prevent it. Thus, animal models may be of considerable value for improving our understanding.

There are different animal models of acute lung injury. The most marked collapse and consolidation of lung can be obtained with repeated saline lavages, for example 10 lavages with a volume corresponding to the FRC.²⁶ This produces an unstable lung that will almost completely collapse if there is no end-expiratory pressure support. However, in this model the lung is easily recruitable and the whole lung may be reopened and well aerated with a positive end-expiratory pressure (PEEP) of 10 to 15 cm H₂O. However, PEEP alone may not reopen a collapsed lung. A recruitment maneuver with inflation of the lung to an airway pressure of 50 to 55 cm H₂O may be required. After the recruitment, the lung can be kept open with more moderate pressures, provided that PEEP has been applied.

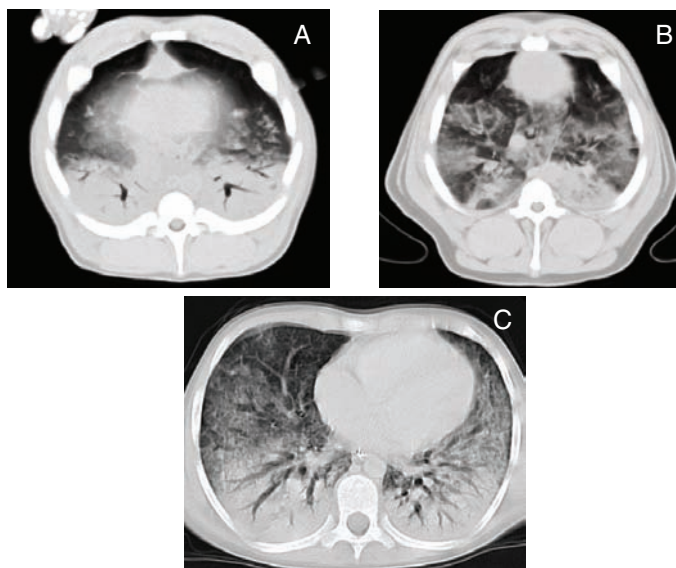


Figure 12.7 CT-scans in two porcine lung injury models (lavage: A, upper left panel; oleic acid injury: B, upper right). For comparison a CT from a patient with severe ARDS is shown (C). Note the widespread distribution of dense and atelectatic regions.

Oleic acid lung damage causes also considerable collapse, and the lung may be more difficult to recruit than after lavage.^{27,28} Endotoxin infusion, perhaps the most faithful model of human ARDS with sepsis, will most often cause a more varied appearance on the CT with both complete collapse or consolidation (fluid-filled alveoli) and reduced aeration.²⁸ Again, full recruitment of collapsed or consolidated tissue may be difficult to obtain in this model. Figure 12.7 shows CT findings in different acute lung injury models.

In human acute lung injury and ARDS, a distinction has been made between primary (pulmonary) ARDS and secondary (extrapulmonary) ARDS.²⁹ The former is the result of an insult to the lung tissue per se, for example pneumonia, aspiration of gastric content (hydrochloric acid), and inhalation of toxic fumes. Extrapulmonary ARDS follows abdominal insults and development of abdominal edema (ascites) and general sepsis. In primary ARDS, a cranial displacement of diaphragm need not occur, and no external forces are applied to the thoracic cavity, whereas in extrapulmonary ARDS, an increased abdominal pressure will displace the diaphragm and contribute to reduced lung volume. Thus, a rather wide range of potential insults to lung integrity exists. These insults can be simulated in different animal models. Primary ARDS can be produced by hydrochloric acid instillation into the trachea and possibly, exposure to toxic fumes. Secondary ARDS will develop with endotoxin infusion and oleic acid injection. There is considerable need to study primary and secondary ARDS in different animal models to explore effects on the lung and optimum treatment modalities.

Individual Lung Ventilation

One-lung ventilation is used during pulmonary surgery; one lung is ventilated to ensure gas exchange and oxygenation of blood, and one lung is nonventilated to enable surgery on it.³⁰ Another situation when one-lung ventilation is used is during unilateral lung pathology.³¹ Aspiration and trauma may affect only one lung, making it stiff and difficult to ventilate, whereas the other one is normal and will receive the bulk or all of ventilation if the patient is ventilated through an ordinary single lumen endotracheal tube. By applying a double lumen endobroncheal catheter, ventilation can be directed to each lung according to what is considered appropriate, and different PEEP levels can be applied. Moreover, locally applied drugs or physical manipulation can modify blood flow distribution to each lung. This is a field of interest for research, and a few words will be said how to provide independent or differential lung ventilation in different animal species.

In humans, the double lumen endobroncheal catheter is designed so that one lumen opens in the trachea before the bifurcation into the two main bronchi (thus above the carina). The other channel can either be directed into the left main bronchus or the right main bronchus. The tube design differs between in these two catheters. A hook can be attached to the tube that will facilitate the positioning of the catheter with the hook riding over the carina. One balloon is inflated in the trachea above the opening of the tracheal lumen so that no air can leak out to atmosphere. The tip of the endobroncheal catheter with the distal lumen will also have an inflatable balloon that secures air tightness for the left or right lung. The other lung will thus be ventilated from the tracheal lumen (Figure 12.8).

In the pig and the dog, which are frequently used in anesthesia research, the tracheobronchial tree is somewhat different from that in humans. Thus, in the dog the lobar bronchi in both right and the left main bronchus depart so close to the carina that it is difficult to inflate a balloon to secure air tightness without occluding the upper bronchus on either side. A specially designed tube, called a Kottmeier tube, enables separation of the two main bronchi.³² This is achieved by using a widebore double-lumen endobronchial catheter with an inflatable balloon at the tip on the rim between the two channels. When inflated, the balloon establishes a tight seal against the carina and separates the channels to the right and left lung (Figure 12.8).

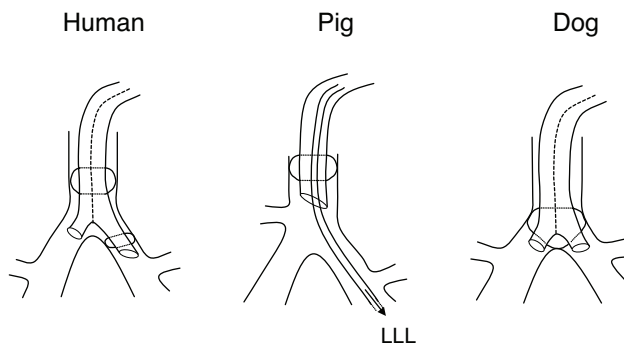


Figure 12.8 Schematic drawing of the upper tracheobronchial tree and the positioning of a double lumen endobronchial catheter in human, pig, and dog. In the human, one cuff occludes the trachea and another cuff the end of the right or left bronchial catheter. This enables separate ventilation of the left and right lungs. In the pig, the right upper lobar bronchus departs from the trachea and makes it difficult to separate the left and right lungs. One technique that has been frequently used by the authors³⁴ is to insert a conventional tracheal tube and through it advance a narrow catheter down to the left lower lobe. By this means separate ventilation can be established between different lung regions. In the dog, the upper lobar bronchi depart so close to the trachea and carina that the design of the tube has to be different, as shown in the figure. This design is often referred to as a Kottmeier tube.

In the pig, the right upper bronchus departs from the trachea above the carina. This makes it difficult to use conventional double lumen endobronchial catheters since the tracheal balloon can obstruct the opening to the right upper bronchus. Strict individual lung ventilation may therefore be difficult. We have ourselves used another approach for the study of different lung regions and the application of different ventilatory techniques to these regions. We advance a narrow bronchial catheter down to the left lower bronchus through the tracheal tube. This enables separate ventilation of the left lower lobe. The right lung and the left upper lobe are ventilated via the tracheal tube and create the other lung compartment (Figure 12.8).³³

THE MECHANICS OF THE RESPIRATORY SYSTEM

Physiology and Pathophysiology

The forces that are applied to the respiratory system will determine the ventilation, whether the forces are generated by the respiratory muscles or by an external ventilator. The impedance to breathing consists of two major components, the elastance of the lung and the chest wall, and the resistance to gas flow in airways and to tissue movement. The inverse of elastance (e), compliance (c), is more commonly used in pulmonary physiology and is thus: $c = 1 \div e$. The mechanics variables, compliance and resistance, can also be used to quantify the severity of lung function impairment and they are often used for diagnostic and prognostic evaluations. The recording of compliance is also used in the guidance of the ventilator setting.³⁴

Compliance is reduced during anesthesia,³⁵ although the mechanisms are not fully understood. The fall in FRC, the formation of atelectasis, and a possible decrease in surfactant function may all contribute to the reduced compliance. Recruitment of collapsed lung increases compliance, reasonably explained by more lung tissue being available for inflation of gas. However, observations in a human study show that this is not the full explanation. Thus, a vital capacity maneuver opened up collapsed lung and increased, as expected, compliance, but during the following hour compliance fell and to the same extent whether lung tissue began to collapse again or whether the lung was maintained open.³⁶ This suggests that another or additional mechanism is of importance, possibly

changes in the surfactant properties. The surfactant function is also of paramount importance from the moment of birth, and deficiency may cause life-threatening respiratory distress, as can be seen in prematurely born children.³⁷ Bovine or artificial surfactant has been used with success to prevent widespread lung collapse and improvement in respiratory mechanics in the neonate.³⁸ Much of the preclinical research has been done in premature lambs.

Resistance will be elevated in the presence of airway obstruction, for example by bronchospasm as in asthma or by destruction of the airway wall as in chronic bronchitis. Various animal models have been used for such studies. Many experiments have been executed in a rabbit model, in which the airway response to different bronchoactive agents is similar to that seen in humans.³⁹ In this model, all pathophysiology characteristic of asthma could be reproduced by the nebulization of saline to peripheral airways without increasing resistance to a measurable degree. In a variation of that model instead employing nebulization of metacholine, bronchodilating effects of inhaled compounds could be studied.^{40,41}

Recording of Mechanics

Measurements of the respiratory mechanics in the anesthesia and intensive care situation mostly include all parts of the respiratory system, chest wall, and lungs. The measurements are relatively simple in the mechanically ventilated subject or animal, since airway pressure and flow measurements provide information for overcoming total impedance. To enable distinction between total respiratory compliance and resistance, end-inspiratory and expiratory pauses are required to produce short moments of zero gas flow. If constant flow is administered during inspiration, the sudden cessation of flow at end-inspiration causes a rapid drop in pressure and this drop divided by inspiratory gas flow will give respiratory resistance.⁴² Similarly, compliance will be calculated as the insufflated volume divided by end-inspiratory minus end-expiratory pressure. Details are given in Figure 12.9. However, things may be more complicated if there is no or too short end-inspiratory pause to enable zero gas flow. It may be even more difficult in the presence of ongoing flow at end-expiration. Airway pressure will then not reflect pressure inside the lung. There will be a pressure difference, commonly called intrinsic or auto PEEP.⁴² There are hints how to detect it. The most reliable technique is to simply occlude the tracheal tube or its connection at end-expiration and measure the pressure distal to the occlusion site. This pressure will increase during the no-flow condition to become equal to alveolar pressure. Such an occlusion maneuver may disclose a large pressure difference with an intrinsic PEEP that may be 10 to 20 cm H₂O higher than the pressure in the airway tubing. Animal models may be helpful in developing techniques that do not need to interrupt the ventilatory pattern but still provide reliable recording of alveolar pressure.

It is often desirable to make measurements of the compliance and resistance of the lung *per se*, separate from compliance and resistance of the chest wall. This will give better resolution of changes in the lungs. Moreover, there is an increasing interest also in the chest wall mechanics, not the least since the coining of “primary” and “secondary” ARDS, as mentioned above.²⁹ Secondary or extrapulmonary ARDS impedes pulmonary function by limiting the volume of the thoracic cavity and the tidal excursion of the lungs, in addition to potential direct effects on the lung tissue. One can therefore anticipate more interest in measuring the chest wall mechanics in future research and clinical monitoring.

The separate recording of lung and chest wall mechanics requires that pleural pressure, or its substitute, esophageal pressure, be measured. This is standard technique in upright awake human subjects and is frequently used for diagnostic and prognostic investigations. While the recording of pleural pressure carries considerable risk and therefore has been abandoned in clinical practice, esophageal pressure recording is common. The esophageal pressure is normally recorded with an esophageal balloon catheter that is swallowed to the lower third of the esophagus. For further details see reference 43.

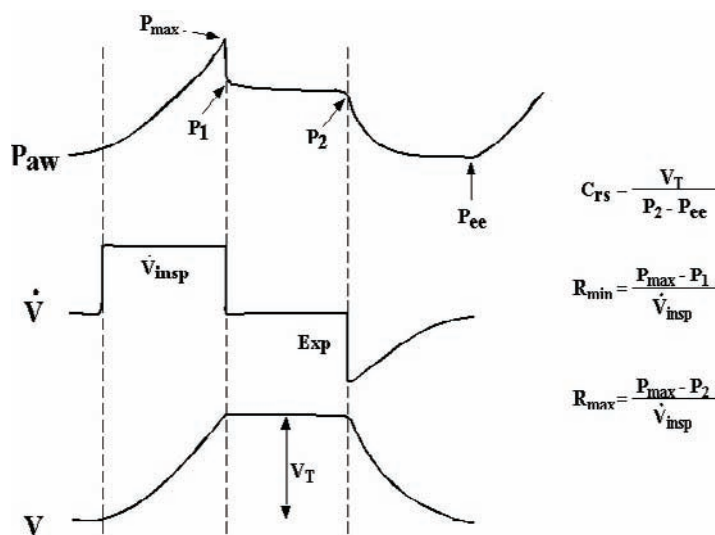


Figure 12.9 Airway pressure (P_{aw}), flow (\dot{V}), and volume (V) curves over a breath delivered by a ventilator. By applying an end-inspiratory pause, the airway pressure will drop from P_{max} to P_1 with a further slow decrease to P_2 during the no-flow interval. Calculation of compliance and resistance is shown in the figure.

The situation is more difficult in the supine subject because the mediastinal organs rest on the esophagus and contribute to the pressure measured inside in the esophagus. Although there have been many attempts to measure esophageal pressure in the supine position and commercial equipment is available, the results are not very reliable. Direct comparison between pleural pressure recordings that might be more acceptable in the animal model and esophageal pressure are highly welcome. However, the recording of pleural pressure is also difficult and can produce considerable methodological error because there is normally a surface liquid pressure caused by the proximity between the visceral and the parietal pleura that are just touching each other, barely separated with a thin fluid layer. When a catheter or a needle is moved into this space, a small volume of gas has to be introduced to enable a pressure recording but this will also affect the original surface pleural pressure. The introduction of a small amount of fluid would more accurately record the initial pleural surface pressure, but the added fluid will move down along the gravitational axis away from the position of the catheter or needle tip.

The most accurate but also complicated technique is to do minor surgery to expose the parietal pleura to atmosphere and then attach a small chamber with a window glass with an airtight seal. By visual or photometric technique it can be seen whether the parietal pleura is protruding into the chamber or away from it. By applying a known pressure in the chamber, the parietal pleura can be repositioned to its initial shape fitting with the curvature of the thoracic cavity. This applied pressure will reflect the fluid pressure at this level.⁴⁴ If two windows are established, vertical differences in pleural pressure can be detected. This difference in pleural pressure will determine the distribution of the ventilation as mentioned in the Introduction paragraphs. Thus, any changes in this pleural pressure difference caused by changes in the lung tissue characteristics or in the chest wall and their consequences for ventilation distribution can be detected. Because of the difficulty in applying invasive techniques in the human subject, animal models can be of importance in the study of respiratory mechanics, with partitioning into different components and with the analysis of regional mechanics.

VENTILATION DISTRIBUTION

The forces that determine the distribution of ventilation have been discussed in the previous paragraph. The assessment of ventilation distribution will require other tools. Most of these are of a noninvasive character and can therefore be employed in human studies.

The ventilation distribution can be studied by the use of radioactive isotopes, either radiolabeled tracers or radioactive gas. Radioactive droplets have been used, but the size of the droplets limits the distribution in the finer branches of the airway tree. To reach the smallest bronchiole the droplet size shall be less than 1 to 2 μm . Ultrasonic nebulizers may provide this droplet size. However, droplets may aggregate during the passage through the airway tree, become bigger and therefore impact more easily on the airway wall. Smaller sizes of the particulate can be obtained by burning a carbon rod in an electric bridge that may produce particle sizes as small as 0.1 μm , so called pseudo-gas. The carbon rod is pretreated with a suitable isotope, usually $^{99\text{m}}\text{Tc}$ (technetium). This has become a standard technique in human investigations.⁴⁵ A reference method is the inhalation of a radioactive gas, $^{81\text{m}}\text{Kr}$ (krypton), that will be distributed down to the alveoli in proportion to regional ventilation.⁴⁶ Due to its short half-life, 11 sec, the isotope will fade away in the alveolar spaces and this is why it will reflect ventilation distribution and not the distribution of gas volume. The short half-life of krypton requires that a generator of that gas is used, ^{81}Rb (rubidium), which has a half-life of 4.6 h. Still, the generator has to be produced in the morning and delivered within a few hours to the laboratory in order to be used for the following 5 to 6 hours before its activity is too low to allow measurements. Another radioactive gas that has been used frequently in previous years is ^{133}Xe (xenon).⁴⁷ It has a much longer half-life, and it will therefore distribute according to the regional alveolar volume. To detect regional ventilation one has to follow the washout of the radioactive gas once its inhalation has been discontinued.

The animal model will enable more invasive study of ventilation distribution. However, it will be limited to the cannulation of larger airways and will thus allow ventilation distribution to different lobes of the lung and between the left and right lung.

PERFUSION DISTRIBUTION

Physiology and Pathophysiology

The gravitational distribution of blood flow in the lung has been mentioned earlier in this chapter. However, in dogs and horses blood flow distribution is more even between upper and lower lung regions than in humans. A higher vascular resistance in the lower, anterior lung regions than in upper regions can explain this.⁴⁸ An animal that is mostly standing on its four legs (prone) may benefit from this uneven distribution of vascular resistance that causes more even perfusion. Humans may not benefit from this since they are moving between different positions, so that one optimum posture cannot be identified.

With an increase in intrathoracic pressure, as with the application of PEEP, the return of blood flow from systemic veins into the thoracic cavity and the right heart is impeded and cardiac output is reduced. Fluid loading of the vascular system and use of vasoactive and cardiostimulating drugs can counter the fall in cardiac output. Moreover, with increase in intrathoracic pressure, blood flow is forced down towards dependent regions, i.e., dorsal regions if the subject or animal is in the supine (dorsal recumbent) position.⁴⁹ A moderate PEEP of 10 cm H_2O applied to both lungs in the lateral position may force all blood flow to the dependent lung with virtually no perfusion of the upper lung.⁵⁰ The distribution of blood flow in the lung is a major determinant of gas exchange, and since blood flow can be manipulated both by pharmacological and mechanical means this area can be of considerable interest for animal research. Techniques to measure blood flow will be dealt with in the next paragraph.

Recording of Blood Flow

Total lung blood flow, normally similar to cardiac output, is commonly measured by indicator dilution techniques. An indicator bolus is injected in a central systemic vein and will be fully mixed with blood when passing through the right ventricle. Its dilution can be detected either in the pulmonary artery or in a systemic artery. The most commonly used technique is thermodilution where ice-cold saline (1 ml in the rabbit, 10 ml in humans, for example) is injected and its dilution detected by a thermistor in the pulmonary artery. The thermodilution curve will be damped during the passage through the lung tissue due to heat dissipation to surrounding tissue, making its detection in the systemic artery less reliable for cardiac output calculation. Another frequently used indicator, indocyanine green (“Cardiogreen”) can be used and be detected in a systemic artery, since there will hardly be any loss of the indicator in the lung.

A golden standard of cardiac output determination has been the “Fick principle on oxygen.” This means that the amount of oxygen taken up per unit time (minute) is divided by the oxygen content difference between systemic arterial blood and mixed venous blood, the latter taken from the pulmonary artery. This technique is highly reliable and reproducible but requires pulmonary artery catheterization and blood sampling for subsequent spectrophotometry for determination of oxygen content. In order to make the determination less invasive and simpler to execute, the Fick principle has also been applied to the carbon dioxide elimination from the lung. Breath holding and rebreathing maneuvers increase the expired CO₂ concentration and enable extrapolation to mixed venous CO₂ concentration. The arterial CO₂ concentration can be set equal to end-tidal CO₂, although large differences may be seen in severe lung pathology. The technique is less accurate than the Fick principle on oxygen.

The regional distribution of blood flow in the lung can be assessed by the injection of markers that are trapped in the pulmonary vascular tree in proportion to blood flow. A standard technique in human clinical investigations is the injection of ^{99m}Tc-labeled macro-aggregated albumin (MAA).⁵¹ The particles are in the size range of 15 to 50 μ m and will therefore lodge in the pulmonary vessels. An ordinary dose contains approximately 300,000 particles in a human investigation. This should be compared with the almost 300 million arterioles in which the particles are lodged. No measurable effect on vascular pressures should therefore be expected.

The technique is noninvasive and therefore suitable for human investigations. By subtracting one image from another, repeated measurements can be performed to study perfusion under different conditions. In practice, this subtraction technique is limited to 3 to 4 measurements, and the radioactive dose has to be increased with each new recording to minimize the noise from previous isotope injections. For animal research, radioactively labeled microspheres are commercially available with different bead sizes and labeled with different isotopes.⁵² Knowing the energy spectrum of each isotope, the distribution of the different isotopes can be detected with gamma camera technique. The lungs can also be taken out after a finished experiment, rinsed with formalin solution, inflated to an airway pressure of 20 cm H₂O and dried for 4 or 5 days. The lungs can then be cut into pieces and each piece analyzed for its radioactivity in a gamma counter. This technique eliminates the need of a gamma camera, and more detailed information might be obtained but the technique is obviously more cumbersome.

Instead of using isotope-labeled microspheres, beads with different colors and fluorescence characteristics may be used.⁴⁷ After formalin treatment, inflation, and drying, the distribution of the beads can be detected with fluorescence light microscopy. Still another technique, based on colored beads, is to extract the color from the piece of lung tissue under study. This technique makes the analysis much simpler. To ensure as much as possible a reliable three-dimensional reconstruction of blood flow distribution the whole lung can be embedded in a paraffin block with well defined x-, y-, and z-coordinates so that when the lung is cut into pieces each piece can be allocated according to this coordinate system. However, a potential source of error with all techniques that

require *ex vivo* analysis on excised lungs is that the lung shape per se might be distorted compared to when the lung was still inside the thoracic cavity.

PULMONARY GAS EXCHANGE

Physiology and Pathophysiology

The gas exchange in the lung is determined mainly by the matching of alveolar ventilation and blood flow (\dot{V}_A/\dot{Q}). A good match results in optimal oxygenation. There is considerable difference between humans and different species. These differences will also be discussed in further detail and compared with humans⁵³ below.

Dog

During the 1960s and 70s the dog was frequently used for anesthesia studies, assuming that the results might be transferable to humans. However, there are differences. The efficiency of oxygenation is slightly decreased in dorsal recumbency during anesthesia.⁵⁴ However, \dot{V}_A/\dot{Q} matching is better than in humans, with a narrow unimodal distribution of \dot{V}_A/\dot{Q} and a small scatter of \dot{V}_A/\dot{Q} ratios (logarithmic standard deviation of lung perfusion, log SDQ). Shunt does not develop, which is the opposite of what occurs in humans.⁵⁵ It has also been shown that atelectasis does not develop, as mentioned above.¹⁹ However, regions with high \dot{V}_A/\dot{Q} develop, in particular when a positive end-expiratory pressure (PEEP) is applied.⁵⁶ This is caused by a tiny perfusion of so-called corner vessels in the uppermost part of the lung.⁵⁵ Bronchial provocation with metacholine in the dog results frequently in an extra mode of low \dot{V}_A/\dot{Q} ⁵⁷ that seems to be explained by well-developed collateral ventilation, see also above.¹²

Pig

During anesthesia the \dot{V}_A/\dot{Q} matching is less efficient than in the dog with larger scatter of \dot{V}_A/\dot{Q} ratios, and the pig does also develop some shunt.⁵⁸ Bronchoprovocation tests with metacholine do not produce low \dot{V}_A/\dot{Q} , contrary to the findings in the dog.⁵⁸ This difference with the dog may be explained by the absence of collateral ventilation in the pig.

Sheep

Sheep have been studied standing in a cradle awake, and they show a broad unimodal \dot{V}_A/\dot{Q} distribution with a much larger scatter than in waking humans.⁵⁹ Sheep have well-developed interlobular recepta that reduce collateral ventilation, and this may explain the poorer \dot{V}_A/\dot{Q} match. There is further slight worsening of the \dot{V}_A/\dot{Q} match during anesthesia in the dorsal recumbent position, and shunt is as large as in anesthetized humans.

Rabbit

In the anesthetized, mechanically ventilated rabbit in dorsal recumbency, a surprisingly large \dot{V}_A/\dot{Q} scatter has been found, not the least in view of the small size of the animal, with a weight of 3 to 4 kg and a vertical lung distance of 7 to 8 cm. However, no or minor shunt was observed.⁶⁰ The rabbit has good collateral ventilation, which would also have prevented poor matching. However, it is possible that there are species differences with regard to hypoxic pulmonary vasoconstriction; the rabbit is well known to easily develop hypoxemia during anesthesia. The rabbit does also develop considerable high \dot{V}_A/\dot{Q} regions on metacholine provocation⁶⁰ and has been frequently used as an asthma model.

Duck

Another animal that has been studied during spontaneous breathing during anesthesia is the duck. This research is worth mentioning because the results did not fit known gas exchange models. Thus, the distribution of ventilation and blood flow in parallel, as in humans and most mammals, had to be replaced by another analytical model in the duck, based on a cross current flow model.⁶¹ It is well known that birds have a parabronchial system where the inspired air follows one system of airways to alveoli and another expiratory airway system back to atmosphere. On its way respiratory air is meeting a blood flow that goes in the opposite direction. This gas exchanging system is highly efficient, and the same principle is used in many industrial applications for heat exchange.

Horse

Standing awake horses (trotters) with a weight of approximately 500 kg showed a surprisingly good \dot{V}_A/\dot{Q} matching, comparable to the healthy awake human,⁶² and no or minor shunt was noticed. In view of the high vertical lung distance of about half a meter, this might seem surprising, not the least when comparing with the poorer matching in standing awake sheep with less than half the vertical lung distance of the horse. Also, the anesthetized rabbit showed considerable mismatch despite much smaller lung distance.

Pulmonary gas exchange is affected also in waking recumbent ponies trained to lie down on their sides on command even though they are not anesthetized.⁶³ The influence of body position on pulmonary gas exchange during anesthesia has been studied in pigs and horses.^{64,65} Arterial oxygenation is better in sternal (prone) position compared to lateral or dorsal recumbency. It has also been shown that the body shape might influence gas exchange during anesthesia in horses.⁶⁶ Round-bellied horses have a lower arterial oxygenation than flat-bellied horses in all body positions. This may be related to the pressure exerted by the abdominal contents on the dependent lung and diaphragm, which should be higher in the round-bellied horse.

During inhalation anesthesia and spontaneous breathing, gas exchange deteriorates.⁶⁷⁻⁶⁹ The main cause is development of a large shunt. The shunt is larger (approximately one third of the cardiac output), and the arterial oxygen tension lower in dorsal compared to left lateral recumbency (shunt one fifth of the cardiac output). Conventional mechanical ventilation in dorsal recumbency reduces cardiac output and does in general not improve gas exchange compared to spontaneous breathing, except for normalization of PaCO_2 . With the horse in lateral recumbency, conventional mechanical ventilation reduces the venous admixture and improves PaO_2 to some extent, but the cardiac output is reduced concomitantly. The fall in cardiac output may be attributed to impeded venous return to the heart by the increased intrathoracic pressure.

Giraffe

Gas exchange was studied in a young giraffe with a weight of 500 kg when it was given a light anesthesia for the application of a plaster in the treatment of a foot fracture. Measurements of the \dot{V}_A/\dot{Q} were made during Fentanyl anesthesia in a sitting position with the head kept up in a cradle. During spontaneous breathing, a good match of the \dot{V}_A/\dot{Q} was seen, better than in anesthetized humans. This is surprising in view of the tall lung height of approximately 0.6 to 0.7 m. Another unexpected finding was an ordinary dead space ventilation of the same size as in the horse. The airway dead space was calculated as less than one liter, and in view of the more than 3-meter trachea, one may have anticipated a larger volume (own unpublished observations). The explanation is that the giraffe has a very narrow trachea,¹¹ reducing dead space but increasing airway resistance in the event of rapid breathing. This is illustrated in Figure 12.10.

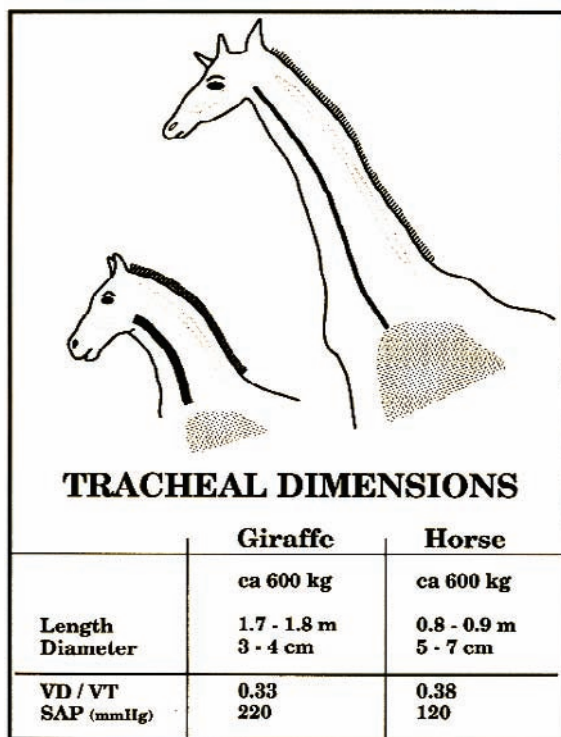


Figure 12.10 Tracheal dimensions in giraffe and horse and the dead space/tidal volume ratio (VD/VT). Note the similar dead space despite different shapes of the neck.

RECORDING OF GAS EXCHANGE

The assessment of gas exchange can be based on a blood gas analysis that provides an overall value (single compartment analysis) or different multicompartment models. The latter will provide better insight into mechanisms underlying impaired oxygenation or CO₂ elimination. They will be dealt with below. For further reading, see Reference 70.

Single-Compartment Analysis

For a given \dot{V}_A/\dot{Q} ratio of the lung, there is a pair of P_aO_2 and P_aCO_2 that match the \dot{V}_A/\dot{Q} for a given inspired O₂ concentration. It also has to be assumed that there is no diffusion impairment. This relation is given in the Fenn and Rahn diagram (Figure 12.11). It can be seen that in a situation where there is ventilation but no blood flow (\dot{V}_A/\dot{Q} of infinity, i.e., dead space), P_aO_2 equals inspired oxygen tension [P_iO_2] and P_aCO_2 is zero. At the other end of the possible range, there is blood flow but no ventilation (\dot{V}_A/\dot{Q} of zero, i.e., shunt), with P_aO_2 equaling mixed venous oxygen tension (P_vO_2) and P_aCO_2 similar to mixed venous carbon dioxide tension (P_vCO_2). In between, unique pairs of P_aO_2 and P_aCO_2 values match all possible \dot{V}_A/\dot{Q} ratios. Thus a blood gas can tell you the average \dot{V}_A/\dot{Q} of that lung, assuming no diffusion impairment. Whether this is useful information may be argued, still it is presented here to help understand the relationships between blood gases and \dot{V}_A/\dot{Q} .

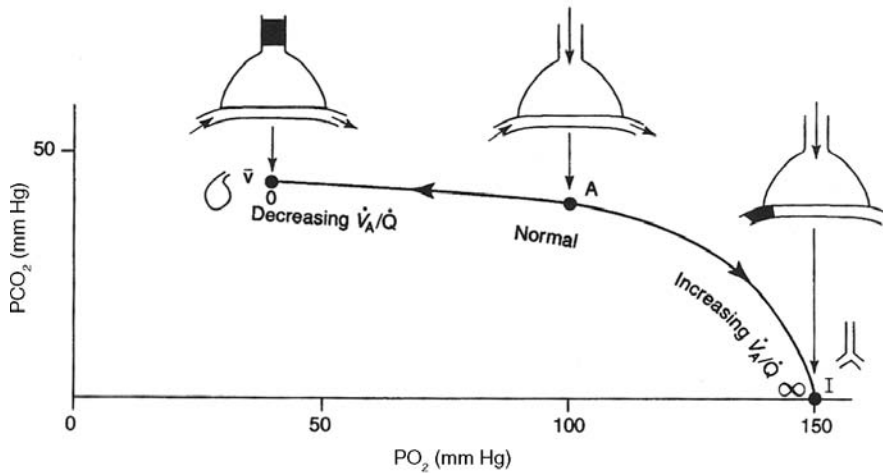


Figure 12.11 Oxygen–carbon dioxide diagram showing the effect of different ventilation–perfusion ratios (\dot{V}_A / \dot{Q} line). Three lung units are shown: shunt (located at the mixed venous point v), normal (ideal) ventilation–perfusion ratio (A), dead space (located at the inspired gas point I).

Three-Compartment Analysis

A more advanced but still simple modeling of the \dot{V}_A / \dot{Q} distribution is the three compartment analysis where one compartment is ventilated but nonperfused (dead space), one is ventilated and perfused (ideal compartment), and one is perfused but not ventilated (shunt) (Figure 12.12). The physiological dead space can be calculated according to:

$$V_D / V_T = (P_a\text{CO}_2 - P_E\text{CO}_2) / P_a\text{CO}_2$$

where V_D = dead space, V_T = tidal volume, $P_a\text{CO}_2$ = arterial PCO_2 , $P_E\text{CO}_2$ = mixed expired CO_2 tension. The $P_E\text{CO}_2$ can be measured by collecting the expired gas in a bag, shaking it to ensure thorough mixing, and then measuring the concentration or partial pressure by gas absorption technique or infrared spectroscopy. The infrared meter also enables continuous, online recording of expired gas, and by multiplying instantaneous PCO_2 with the continuously measured expired gas flow and integrating the signal over time, $P_E\text{CO}_2$ is obtained.

Shunt can be assessed from the oxygenation of blood and is determined by an equation that is identical in design to that of dead space:

$$Q_s / Q_t = (C_c\text{O}_2 - C_a\text{O}_2) / (C_c\text{O}_2 - C_v\text{O}_2)$$

where Q_t = cardiac output, Q_c = blood flow through ventilated lung tissue, Q_s = shunt, and C_a , C_c , and C_v = concentration (of O_2) in arterial, end-capillary, and mixed venous blood, respectively.

Multicompartment Analysis

By using several gases with different solubility in blood, a more detailed analysis of \dot{V}_A / \dot{Q} can be made. Thus, Wagner and coworkers^{71,72} developed a multiple inert gas elimination technique

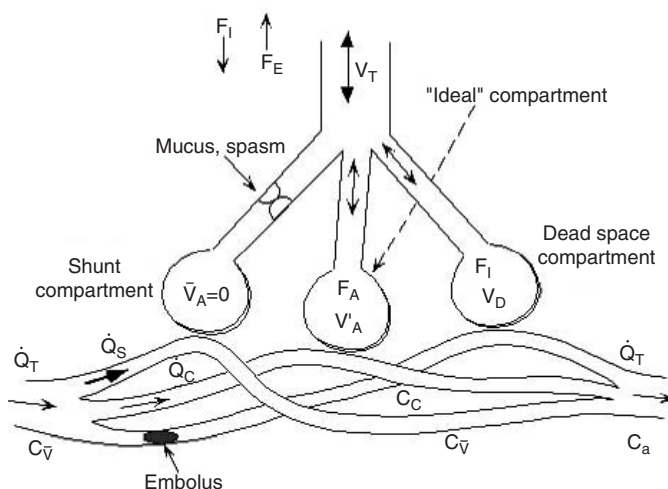


Figure 12.12 Three-compartment analysis of gas exchange. One compartment is ventilated but not perfused (dead space), one is ventilated and perfused (the ideal compartment), and one is perfused but not ventilated (shunt). In practice, however, there is no sharp distinction between compartments. There are units that are ventilated in excess of the perfusion, and they will be incorporated in both the dead space compartment and the ideal one. Other units are perfused in excess of the their ventilation, and they will be located in both the shunt compartment and the ideal one. The distinction between the compartments will depend on the solubility of the tracer gases that are used. The use of O_2 for the assessment of shunt will not enable calculation of "true shunt" but will also include regions with "low" \dot{V}_A/\dot{Q} . The "oxygen shunt" might be better described as venous admixture. By the same token, dead space, as assessed by standard CO_2 elimination techniques, will include regions with high \dot{V}_A/\dot{Q} ratios. Finally, the "ideal" compartment will include regions with \dot{V}_A/\dot{Q} ratios ranging from low to high and not only compartments with a \dot{V}_A/\dot{Q} ratio of 1.0.

(MIGET) that is based on the retention and elimination of several (in practice six) "inert" gases (gases obeying Henry's law, i.e., showing a linear relationship between partial pressure and concentration in blood, e.g., SF_6 and xenon) with different solubility in blood. The retention and excretion values for the six gases can be used in order to allocate ventilation and blood flow to a number of hypothetical compartments (e.g., 50) ranging from shunt ($\dot{V}_A/\dot{Q} = \text{zero}$) over compartments with low, normal, and high \dot{V}_A/\dot{Q} ratios to dead space ($\dot{V}_A/\dot{Q} = \text{infinity}$). A schematic drawing of the basic principles is shown in Figure 12.13 and some examples of \dot{V}_A/\dot{Q} disturbance in Figure 12.14. Note that the contribution to the overall retention value from the different compartments has to be weighted by their individual blood flows. As can be understood, the equation is underdetermined, i.e., there are more unknowns than knowns. A number of combinations of ventilation and perfusion values can fit the measured retention and excretion values. However, the single solution that is obtained by the mathematical analysis has been shown to give a reliable description of the \dot{V}_A/\dot{Q} distribution.

Scintigraphic Techniques

The ventilation and perfusion distributions can also be assessed by isotope techniques that can provide a spatial resolution. They have been dealt with separately in the paragraphs on ventilation distribution and perfusion distribution, respectively. By combining them, the distribution of \dot{V}_A/\dot{Q} ratios can be determined. For further details, see Reference 73.

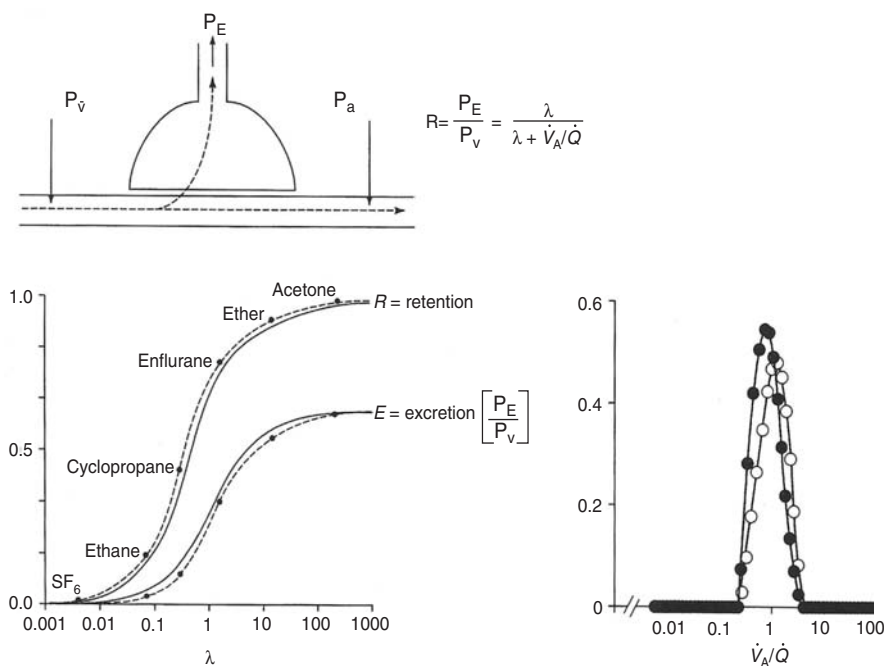


Figure 12.13 Principles of multiple inert gas elimination technique (MIGET).

Upper panel: Gas dissolved in blood will be partly eliminated via the airways when entering the pulmonary capillary. How much is retained in arterial blood is linearly related to the solubility (or the blood:gas partition coefficient) and inversely related to the \dot{V}_A/\dot{Q} ratio. Thus, gas with a high solubility in blood will be retained more than gas with low solubility, and regions with low \dot{V}_A/\dot{Q} ratio will increase the retention of the gas, compared with regions with higher \dot{V}_A/\dot{Q} ratios. (Shunt will cause all gas to be retained.)

Lower left panel: Retention (R) and excretion (E) curves fitted to the measured values of six infused gases with different solubilities in blood. The broken curve has been fitted to the measured values according to the least squares method. The solid curve shows the retention and excretion in a hypothetical lung that is uniformly ventilated and perfused with a \dot{V}_A/\dot{Q} ratio equal to the mean \dot{V}_A/\dot{Q} of the lungs under study. The retention of the least soluble gas is near zero in the normal lung and the other gases are increasingly retained, in proportion to their value. Acetone is retained to almost 100%. The excretion ratios are lower than for the retention, an effect of the mixing with dead space gas. In the normal lung the measured and ideal R and E curves are close to each other.

Lower right panel: The derived \dot{V}_A/\dot{Q} distribution from the retention and excretion data. A good match between ventilation (o) and perfusion (●) can be seen, centered on the \dot{V}_A/\dot{Q} ratio 1.0.

With positron emission tomography (PET) the local blood flow, ventilation, ventilation-perfusion ratios, permeability to proteins, density of receptors, and many other biologically relevant aspects may be estimated *in vivo*. Dynamic processes may be investigated with PET. The technique is based on principles developed for conventional x-ray computed tomography and measures the distribution and biologic behavior of a variety of compounds labeled with positron-emitting isotopes such as ^{15}O , ^{11}C , ^{18}F , or ^{13}N ⁷⁴. These positrons (positively charged antiparticles of electrons) typically have half-lives of a few seconds up to a few minutes and have to be produced with a cyclotron at the investigation site. This makes the access to PET limited.

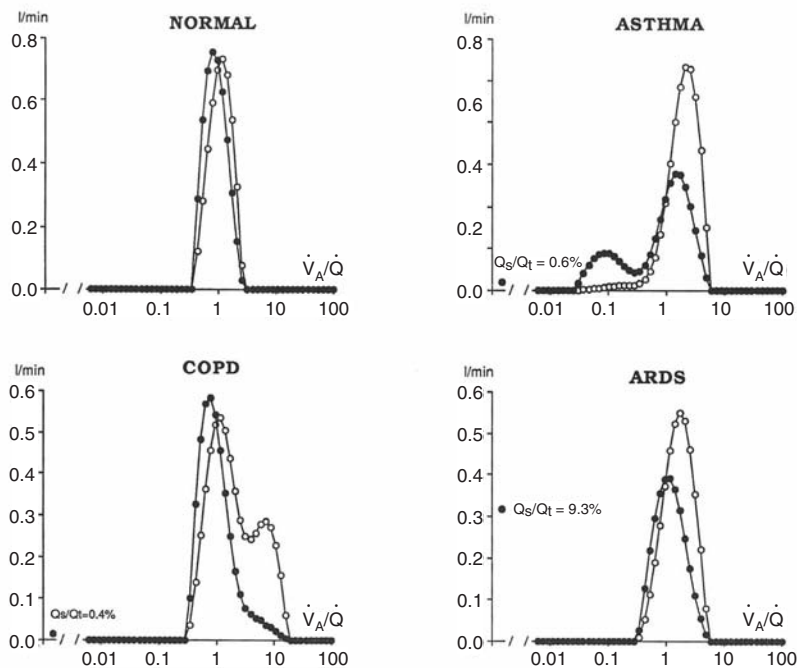


Figure 12.14 Typical \dot{V}_A/\dot{Q} distributions in a normal human, a patient with asthma, a patient with chronic obstructive pulmonary disease (COPD), and a patient with acute respiratory distress syndrome (ARDS). Note the good matching of ventilation and blood flow in the normal subject and the presence of low \dot{V}_A/\dot{Q} mode in the asthmatic, fitting with impeded ventilation in some regions or collateral ventilation (see also Figure 12.5). The COPD patient shows regions with high \dot{V}_A/\dot{Q} , suggestive of reduced perfusion in some regions, possibly an effect of hyperinflation. Chronic obstructive lung disease may also demonstrate low \dot{V}_A/\dot{Q} as in asthma. Finally, the ARDS patient shows a broadened \dot{V}_A/\dot{Q} distribution indicating less good matching of ventilation and blood flow (increased log SDQ). In addition, there is an increased shunt that can exceed 50% in the worst cases.

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CHAPTER 13

Animal Models for the Study of Pulmonary Edema

Göran Hedenstierna and Claes Frostell

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INTRODUCTION

The lung may react with edema and consolidation in response to various insults. The morphological changes are accompanied by functional disturbances signified by impaired ventilation–perfusion matching and shunt with subsequent impediment of oxygenation of blood and carbon dioxide removal. This chapter will review theoretical aspects and animal models of pulmonary edema formation as well as techniques for the assessment of edema formation.

THEORETICAL ASPECTS OF PULMONARY EDEMA

Basic Principles of Edema Formation

About a century has passed since Starling presented experiments demonstrating that hydrostatic and oncotic factors balanced each other over the capillary membrane, determining the rate of fluid moving from the circulation to an extravascular region. A later modification of his work led to the equation now named after him:¹

$$Q = K_f ((P_{mv} - P_{pmv}) - \delta(\pi_c - \pi_t))$$

where Q is the net fluid filtration from the pulmonary micro vascular (intravascular) capillary system to the peri-microvascular (extravascular) pulmonary tissue; K_f is the capillary filtration coefficient; P is the hydrostatic pressure in the micro vascular (P_{mv}) and peri-microvascular (P_{pmv}) compartments, respectively; δ is the protein reflection coefficient (varying between 0 [freely permeable to proteins] and 1.0 [impermeable to proteins]) and π is the protein colloid osmotic pressure in the micro vascular (π_c) and peri-microvascular (π_t) tissue. The expression K_f reflects the surface area available for fluid flux and the hydraulic conductance for fluid.

It is obvious from this expression that edema may form in a tissue for a variety of reasons or due to a combination of factors. In the lung we recognize “hydrostatic” pulmonary edema (HPE) as opposed to “high-permeability” pulmonary edema (HPPE). The idea here is that the former may be a result of mainly extra-pulmonary factors such as left heart failure. In contrast, the latter edema type is mainly caused by an alteration of the permeability of the capillary membranes in the lung, permitting fluid and large molecules to extravasate even at normal vascular pressures. Naturally the therapeutic approach, apart from symptomatic support, differs according to the etiology of edema formation.

Furthermore, edema may accumulate due to a combination of altered hydrostatic and permeability factors. This can e.g., be seen in early experimental septicemia caused by injecting live bacteria or lipopolysaccharide. Sheep typically react with both a sharp increase in pulmonary artery pressure and an increased permeability in lung capillaries.² Both of these factors therefore contribute to edema formation in the lung.

Effects of Airway Pressure or High Capillary Pressure

To what degree high airway pressure is or is not detrimental has been the subject of considerable debate. Since mechanical ventilation (MV) was introduced for the treatment of acute lung injury (ALI, and its most severe form, acute respiratory distress syndrome, ARDS) in the late 1960s, it was obvious that barotrauma with or without pneumothorax was an adverse effect to consider when applying high airway pressure.³ Kolobow and coworkers studied sheep treated with MV and had their lungs overexpanded by the application of peak airway pressures at 50 cm H₂O.⁴ They showed that ALI/ARDS could develop by such high airway pressure even in initially healthy lungs. Webb and Tierney created impressive alveolar edema in rats by ventilating with high airway pressures for only 20 min.⁵ This work was confirmed and extended by Dreyfuss et al.,⁶ suggesting the term “volotrauma” as opposed to barotrauma. This latter study offered evidence that overexpansion of lung tissue by volume is more damaging than pressure alterations. West and Mathieu-Costello have published impressive electron microscopic evidence of how overexpansion by pressure, either of the airway or the capillary vessels (increased blood pressure), will result in “stress failure” of these structures.⁷ The damaged structures allow fluid, larger molecules, and sometimes whole blood corpuscular elements to exit the circulation and participate in edema formation.

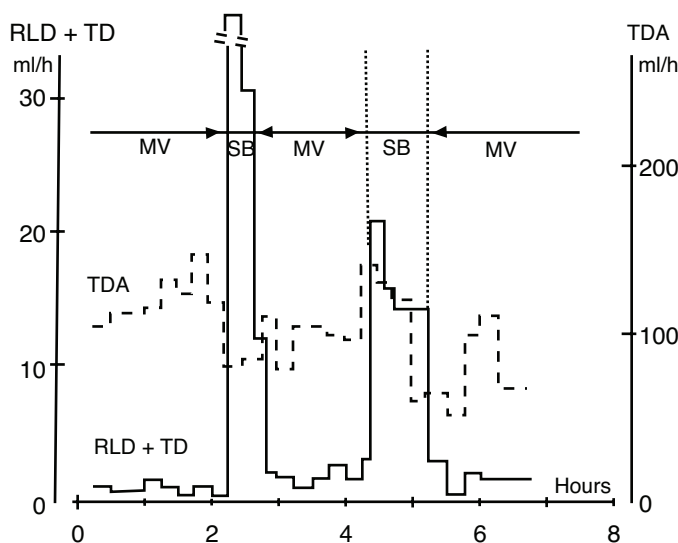


Figure 13.1 Pulmonary lymph flow (RLD + TD) and abdominal lymph flow (TDA) during mechanical ventilation (MV) and spontaneous breathing (SB) in a 30-kg anaesthetised dog. Note the dramatic increase in lung lymph flow during spontaneous breathing, suggestive of impeded lymph drainage during mechanical ventilation. Note also the decrease in abdominal lymph flow during spontaneous breathing. Whether this reflects decreased capillary leakage remains to be shown. *Source:* Frostell, C. et al., *Acta Anaesthesiol. Scand.*, 31, 405, 1987. (With permission.)

Our group measured lung lymph flow in anesthetized dogs ventilated with or without positive end-expiratory pressure (PEEP).⁸ During MV with PEEP, 10 cm H₂O the lymphatic flow from the lung was reduced almost 50% compared to zero end-expiratory pressure (ZEEP): on the other hand, during spontaneous breathing, lung lymph flow was markedly elevated (Figure 13.1).⁸ This indicates that intrapulmonary pressure can influence lung fluid balance and that spontaneous breathing considered in relation to lung lymph flow might have an advantage when compared with MV.

The trauma to the lung that can be produced by high airway pressure has led to the concept of “protective ventilation” with a low tidal volume of 6 instead of 12 to 15 ml/kg bodyweight and a low PEEP of approximately 5 to 10 cm H₂O.⁹ Increased survival in ARDS has been reported,⁹ but doubts exist whether low-pressure technique is free from trouble. Widespread atelectasis and consolidation of the lung are consequences of reduced airway pressure that cause hypoxemia and that may facilitate spread of the inflammatory state.

It can be summarized that in the early stages of lung injury a number of factors contribute to edema formation:

1. Increased capillary pressure (fluid overloading, left heart failure)
2. Elevated airway pressure causing barotrauma (mechanical ventilation, PEEP, need of higher tidal volumes due to intrapulmonary shunting)
3. Elevated airway pressure impeding lymphatic drainage of lung tissue and possibly pleural cavities
4. Increased permeability of the capillary or alveolocapillary membranes
5. Impeded active transport of water from the alveoli back to the interstitium and circulation
6. Extravasation of activated neutrophils and macrophages that further lowers permeability after releasing inflammatory mediators locally and into the bloodstream
7. Micro-thrombotization of lung blood or lymph capillaries by local activation of platelets, reducing the functional area of the lung capillary bed, which contributes to an elevated micro vascular (hydrostatic) pressure (flow limitation) and possibly impedes lymphatic flow

Clearance of Pulmonary Edema

Clearance of extravasated fluid from the lung is a very complex situation. From a clinical point of view, it seems advisable on the basis of the above discussion to keep vascular pressures low (pulmonary artery pressure, central venous pressure). We attempted to optimize Starling factors by using a hyperoncotic hypertonic infusion during pulmonary edema in dogs but found to our surprise that this manipulation did not increase the rate of reduction of extravascular lung water.¹⁰ Obviously, other factors limited the maximum rate of reabsorption of fluid. At this point we consider the Starling equation unable to express by itself all relevant factors that determine lung fluid balance. An illustration of the need to take additional factors into account is the demonstration that the pleural cavities may act as safety factors delaying and minimizing edema formation. It is now known that a significant portion of interstitial fluid during edema formation is excreted into the pleural cavities and cleared from the parietal pleura.¹¹ Experiments on dogs demonstrate to what extent this animal is dependent upon an intact lymphatic system to clear the pleural cavity of protein-rich extravasated fluid. This was shown by ligating the lymphatic vessels draining the pleural cavity, measuring the rate of removal of indicator-labeled protein from a pleural effusion.¹² The dog could not remove such an effusion after lymphatic ligation. Another factor still difficult to assess in terms of functional importance is the discovery of membrane channels for the active transport of water, so-called “aquaporines.” In a recent paper, this topic was reviewed and it was stated that these channels indeed do have unique tasks to fulfill in lung tissue but noted that their absence in knockout mouse strains did not cause lethal physiological impairment or severe illness.¹³

Our overall conclusion must be that a number of factors concerning clearance of edema, as well as safety factors against edema formation, have to be taken into consideration when discussing the fluid balance of lung tissue. We have previously suggested the calculation of “net fluid leakage” per time unit as a more relevant parameter to use for such an analysis.¹⁴

Our understanding today is that there exist several ways by which the lung can rid itself from edema. Fluid can be:

1. Reabsorbed through the capillary membrane by Starling type mechanisms or actively pumped out of the alveoli¹⁵
2. Cleared through the lymphatic system as lymph and returned to the circulation¹
3. Excreted to the pleural space directly from the lung tissue¹¹ and there taken up into parietal pleural lymphatics for return to the circulation
4. Transported to the lung hilus interstitially and from there migrate into the mediastinum and be absorbed¹⁶
5. Cleared through the airway

Different pathways of fluid transport in the lung are shown schematically in Figure 13.2.

GAS EXCHANGE IN PULMONARY EDEMA

If severe enough, edema formation in the lung progresses from interstitial to alveolar edema. It has been stated that alveolar edema starts to form at or above an extravascular lung water value (EVLW) of 12 ml/kg.¹⁷ In early pulmonary edema up to this point, little or no disturbance of gas exchange is to be expected. The lung is a little stiffer (or less compliant) as the interstitium becomes more and more filled with fluid. After this point, gas exchange is progressively impaired as EVLW is elevated. Clinically, the patient displays increased frequency of breathing and some degree of hypoxemia. Retention of carbon dioxide is a late phenomenon. Blomqvist and coworkers attempted to correlate physiologic changes with the level of EVLW determined by indicator dilution in anesthetized dogs during the formation of hydrostatic pulmonary edema.¹⁴ No parameter closely reflected the level of EVLW. Even the degree of hypoxemia could vary greatly depending upon the

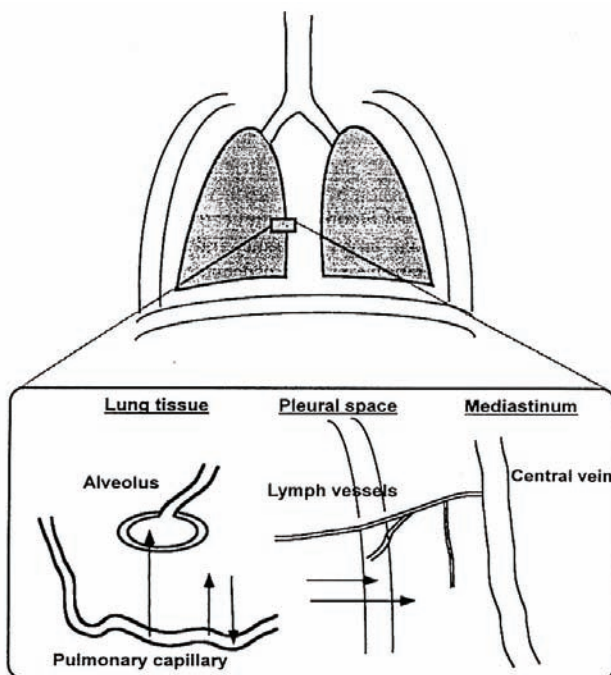


Figure 13.2 Pathways of filtered fluid from the intravascular to the extravascular spaces in the lung. *Source:* Blomqvist, H., Frostell, C., Pieper, R., and Hedenstierna, G., *Acta Anaesthesiol. Scand.*, 34, 370, 1990. With permission.

level of PEEP administered. Thus, intrapulmonary shunting could be almost abolished by PEEP without a reduction in EVLW.¹⁸

MEASUREMENT OF PULMONARY EDEMA

The amount of edema formed in the lung can be measured as the content of blood-free water outside the circulation (= extravascular lung water; EVLW). This way of defining EVLW does not permit a distinction between alveolar, interstitial, and intracellular water. EVLW can be estimated in several ways.

Gravimetry

The “gold standard” has traditionally been determined postmortem by a gravimetric technique, gravimetric EVLW. Obviously, this can only be done once. Excised lung tissue is homogenized after adding water, causing total hemolysis. Residual blood content is estimated by determining the fraction of hemoglobin in the homogenate. Fractional water content is measured by drying the samples to constant weight, and in the end EVLW can be calculated.¹⁹ Normal values obtained for healthy lung tissue are around 4 ml/kg body weight. Interstitial edema develops into alveolar edema in some lung regions at around 12 ml/kg, and values of 20 to 30 ml/kg are typically measured in severe pulmonary edema.¹⁷

A more simple way of estimating edema of excised lung tissue is just to weigh the tissue sample promptly when it has been excised and then again when it has dried to constant weight. The wet/dry weight ratio will then give an indication of the degree of edema. However, blood leak and pooling are confounding factors.

Indicator Dilution Techniques

Typically, two indicators are administered in a central vein, mixed with the blood when passing the heart, and then detected on the arterial side after passing through the pulmonary circulation. In this way the mean transit time for each indicator can be determined. For further details, see reference 20. A source of error with this technique is occlusion of pulmonary vessels, which leads to underestimation of EVLW.²¹

In systems using the so-called thermal/dye-dilution technique, indocyanine green (ICG) is typically used as the intravascular marker. ICG binds to albumin and therefore remains in blood even during lung injury with increased permeability. It will mix with blood in the pulmonary vessels, in the heart, and in the vessels between the lung and the sampling site. It thus gives an estimate of the “central blood volume,” and part of it is pulmonary blood volume. Important information on the vascular filling can be obtained from this variable. It appears to be more valuable in determining whether the patient is hypo-, normo-, or hypervolemic than is any vascular pressure recording,²² e.g., the recording of wedge pressure. Near ice-cold isotonic saline or glucose is used as a “negative heat” indicator that is not limited to the circulating blood but also penetrates the surrounding tissue. The “negative heat” or “cold” diffuses more rapidly into the lung tissue than any other potential marker of the extravascular space would (Figure 13.3). These indicators have been measured either intravascularly in the arterial system with catheter-placed sensors or withdrawn into an extracorporeally placed cuvette and there detected.²³ If the indicators are measured at different sites, e.g., the heat intravascularly and the green dye in a cuvette, it is important to correct for the delay of the green dye signal. Another potential source of error is inability to correctly detect the onset of the dilution curve. Since the curves have less amplitude at high cardiac output because of greater dilution, such an error may become more prominent at high flow than at low. A combination of these sources of error may be the explanation for the cardiac output dependency that has been reported for a frequently used commercial system.²⁴ A later system employs a fiber optic thermistor-tipped arterial catheter, detecting both indicators intravascularly. This system does not display the same cardiac output dependency.²⁴

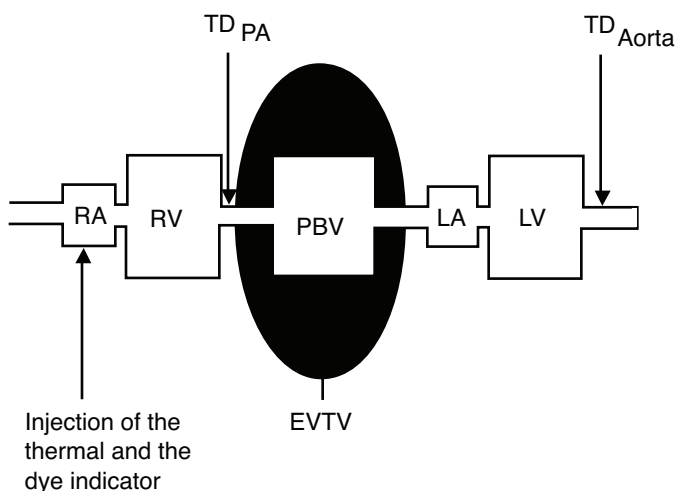


Figure 13.3 Schematic drawing of the cardiopulmonary system in the recording of central blood volume and extravascular lung water. RA = right atrium; RV = right ventricle; PBV = pulmonary blood volume; EVTV = extravascular thermal volume; LA = left atrium; LV = left ventricle; TD_{PA} = thermodilution measurement in the pulmonary artery; Td_{Aorta} = thermodilution measurement in the aorta. The indicator dye is determined simultaneously at the corresponding point in the descending aorta.

In a system using deuterium and dye, arterial blood is withdrawn into an extracorporeal cuvette, which allows for real-time detection of both indicators.²⁵ A possible source of error in any thermistor-based system is the response time characteristics of the thermistor probe, which does not allow for real-time measurements and instead requires assumptions or mathematical corrections. This has been suggested as an obstacle to obtaining reliable EVLW measurements with a thermistor-based technique.²⁶

Radiological Techniques

Conventional X-Ray

The radiological features on a plain chest film permit relatively early detection of edema and a certain quantification.²⁷ By using a “reading table,” an increased level of objectivity can be produced.²⁸ Experienced radiologists may be able to differentiate between different causes of edema by looking at the following features:²⁸

1. Distribution of edema
2. Peribronchial cuffing
3. Airbronchograms
4. Septal lines
5. Vascular pedicle width
6. Azygos vein width
7. Pulmonary blood volume
8. Pleural effusions
9. Blood flow distribution
10. Soft tissue thickness
11. Cardiac size
12. Lung volume²⁸

However, the correlations between edema evaluation from plain chest radiographs and other techniques such as indicator dilution have not always been good.

Computed Tomography

Computed tomography (CT) of the lungs allows a further analysis of the distribution of pulmonary edema and also a global quantification if the CT covers the whole lung. The basic principle is that the X-ray tube rotates around the body during a continuous exposure. A transverse image can then be reconstructed, usually in a 512×512 matrix. Each picture element (pixel) in the matrix will receive an attenuation number on a scale that ranges from -1000 Hounsfield units (HU) (air) via 0 HU (water) to $+1000$ (bone). Collapsed lung tissue or flooded alveoli with no remaining air will thus have attenuation number of approximately 0 HU. Poorly aerated tissue with e.g., 20% air at 80% tissue corresponds to -200 HU. Well-aerated tissue will be found in the range of -500 to -900 HU (a lung region with 70% air corresponds to -700 HU) and overexpanded or hyperinflated lung regions have an attenuation value of -900 HU or lower. Aeration decreases down the lung so that the uppermost regions may have 90% air and the lowermost barely 50% . By knowing the volume of a single pixel (normally a few milliliters) and summing up the gas and tissue volume of each pixel, the gas volume (Functional residual capacity [FRC] if measured after a normal expiration) and tissue volume can be estimated. The tissue volume will thus comprise blood, dry tissue, and extravascular fluid. By comparing the tissue volume in a patient with suspect pulmonary edema with normal values, the excess tissue or edema can be determined.^{29,30} An example is given in Figure 13.4.

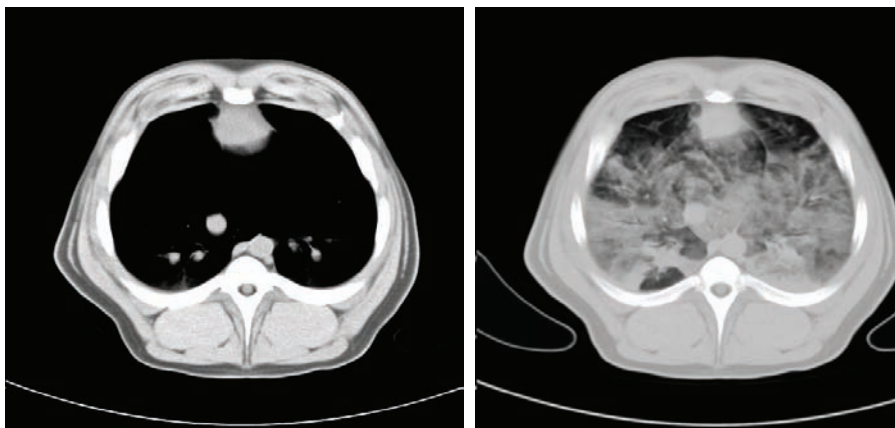


Figure 13.4 CT scans of a pig at baseline (left panel) and after the formation of severe oleic acid–induced pulmonary edema (right panel). A few vessels can be seen in the lower, dorsal region at baseline, as well as a tiny atelectasis in the bottom of the right lung. After oleic acid administration, most of the lung fields are grey-white, indicating edema. Compare with Chapter 12, Figure 12.7C, the CT from a patient with severe acute respiratory failure (ARDS).

It has been assumed that the sequence of fluid accumulation during acute pulmonary edema is quantal, i.e., that an alveolus can exist only in a state of being either air filled and expanded, or fluid filled and collapsed.³¹ Wegenius and coworkers tested this assumption by analyzing CT scans of the chest in pigs on inspiratory and expiratory breathholding at various degrees of oleic acid pulmonary edema.³² The ratio of the mean attenuation on inspiration to that on expiration was calculated and defined as an alveolar instability index. This index correlated well with EVLW measured by the double-indicator dilution technique ($r = 0.98$). The technique may be cumbersome in clinical practice, but the good correlation with the indicator technique supports the hypothesis of quantal behavior of the lung.

Ground glass opacification has frequently been used as a sign of early pulmonary edema. Morphologically, ground glass opacification corresponds to alterations in lung parenchyma that are below the spatial resolution of a CT scanner. In pulmonary edema, it is caused by increased fluid volume in either the interstitial or the alveolar compartment of the lung parenchyma, or in both. A close correlation between the appearance of ground glass opacification on thin-slice high resolution CT and a rise of pulmonary capillary pressure above critical pulmonary capillary pressure has been demonstrated.³³ The critical pulmonary capillary pressure was calculated from the colloidal osmotic pressure (critical pulmonary capillary pressure = $1.55 \times \text{colloidal osmotic pressure} - 6.8$ [pressure expressed in mmHg]).

It may thus be concluded that CT strengthens the subjective evaluation of pulmonary edema as compared to conventional chest X-ray. More important, it enables a quantitative evaluation of excess tissue that in most cases will reflect edema.

Magnetic Resonance Technique

Magnetic resonance (MR) imaging can be used to evaluate the lung water content qualitatively and quantitatively. However, the application of MR to the lung parenchyma has been hampered by three factors:

1. Low proton density resulting in a low signal-to-noise ratio
2. Signal loss due to physiologic motion (cardiac and respiratory movement)
3. Susceptibility artifacts because of multiple air–tissue interfaces

However, there is also the advantage of different spin and relaxation characteristics of different edema formations. Thus, the so-called T1 relaxation time was significantly longer in hydrostatic pulmonary edema than in controls, whereas the T2 relaxation time was not different. This differs from permeability pulmonary edema, where both T1 and T2 relaxation times were significantly longer. Good correlations between T1 and T2 and the extravascular lung water in rats as well as in isolated human lungs have been demonstrated. It may thus be concluded that MR offers unique possibilities to study the distribution of edema, to quantify the amount, and even analyze causes of the edema. However, at present the availability of the technique is limited. For a review, see Reference 34.

Isotope Techniques

Various markers have been labeled with isotopes to quantitate the severity and identify the cause of pulmonary edema. The difference in the distribution volumes between one marker that can diffuse from the vascular into the extravascular space and another marker bound to the vascular space will yield the extravascular lung water (EVLW). The measurement is thus based on the same principle as for thermal dilution. ^{125}I odine-antipyrine or ^{125}I odine has been used to estimate the total and extravascular lung water, and $^{99\text{m}}\text{Tc}$ chnetium-labeled red cells or ^{125}I odine-albumin have been used to evaluate pulmonary blood volume. These isotopes can be detected by a standard gamma camera, and correction for uneven attenuation by body tissue and varying distance to the radiosensitive crystal can be made by an external ring of radioactive source that produces a transmission scan.³⁵

Even more advanced and accurate measurements can be made by positron emission tomography (PET), which allows measurements of region blood volume, blood flow, and EVLW using intravenous H_2^{15}O and inhaled CO^{15}O .³⁶ However, a complicated and expensive technique and limited availability make isotope measurements for the assessment of lung water of limited interest.

The strength of isotope techniques may rather be the possibility to detect changes in pulmonary microvascular permeability. This can be achieved by labeling small proteins with an isotope measuring the accumulation of intravenously injected substances in the lung tissue by external counters or a gamma camera. $^{99\text{m}}\text{Tc}$ chnetium-labeled albumin or $^{113\text{m}}\text{In}$ dium-labeled transferrin have been used in different studies. Interested readers may refer to a comprehensive review by Groeneveld.³⁵

Impedance Measurements

Transthoracic monitoring of the impedance of the thorax is a technique that may detect accumulation of fluid in the lung, whether the fluid is intra- or extravascular.³⁷ A drawback with this technique has been large variations already at baseline and a dependence on the distribution of edema. The impedance will also depend on the gas volume in the lung, so any change in FRC, e.g., by the application of PEEP, will change the impedance. Another problem with the technique is a zero drift that is, at least in part, due to variation in the skin-to-electrode impedance. This has been claimed to be overcome by applying an algorithm that subtracts the skin-to-electrode impedance from the remaining impedance, which is called internal thoracic impedance. Commercial equipment has been developed that includes this algorithm.³⁸ It may be concluded that the recording of changes of fluid accumulation in the lung by thoracic impedance measurements rests on a rather simple and sound principle. The use of the same equipment for assessing stroke volume and cardiac output requires many more assumptions that will render the result much less reliable.

The thoracic impedance can be measured with a few electrodes applied to the chest in order to give a single impedance value. The technique has been developed further, and transverse images of the thorax can be reconstructed. The technique is based on the application of 16 electrodes attached on the thoracic circumference, and by applying a current to varying pairs of electrodes

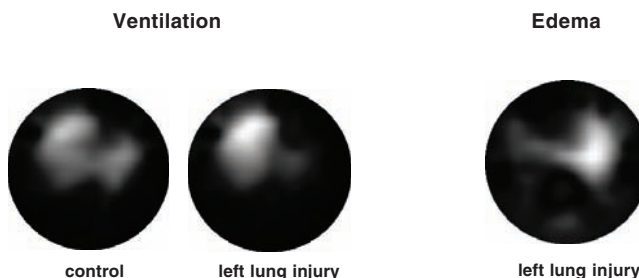


Figure 13.5 Functional EIT images of regional lung ventilation before and after oleic acid-induced left lung injury (left and middle panels), and the distribution of edema between the “healthy” right and the injured left lung (right panel). The pig was mechanically ventilated, tidal volume was 500 ml, and a PEEP of 5 cm H₂O had been applied. Previously unpublished figures, based on the material presented in Frerichs, I., Hahn, G., Schröder, T., and Hellige, G., *Intensive Care Med.*, 24, 829, 1998. Inez Frerichs has generously provided the figures.

and using the other ones for voltage measurements, a cross-sectional reconstruction of the distribution of the impedance can be made.³⁹ The distribution of fluid can thus be assessed. Moreover, by repeating the measurements rapidly over the breath, the distribution of the tidal volume can be evaluated. This enables the detection of nonaerated consolidated lung regions, which makes the electric impedance tomography (EIT) an interesting technique with the potential of clinical value.⁴⁰ Examples are shown in Figure 13.5.

SUMMARY

Formation of pulmonary edema continues to be an important and life-threatening clinical condition. The resolution of hydrostatic pulmonary edema is often rapid but to some extent dependent upon extrapulmonary factors such as heart and kidney functions. High-permeability type pulmonary edema resolves more slowly and is characterized by a complex inflammatory process in the lung. The prognosis is less favorable and the syndrome requires the application of an array of diagnostic and therapeutic resources. It is obvious that understanding of the mechanisms of edema formation, and the resolution of edema, is a prerequisite for successful treatment. Animal models have played an important role in the understanding of edema formation and resolution.

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